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Evolutionary Patterns in Deep-Sea Mollusks

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EVOLUTIONARY PATTERNS IN DEEP-SEA MOLLUSKS

A Dissertation Presented

by

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ABSTRACT

EVOLUTIONARY PATTERNS IN DEEP-SEA MOLLUSKS

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The evolution of deep-sea fauna is poorly known. We know little about the scales of population differentiation, radiation and colonization of the deep sea. This dissertation explores evolution in the deep sea at the level of population differentiation on ocean-wide scales in a gastropod species Benthonella tenella and phylogeny of a deep-sea subfamily of protobranch bivalves, Ledellinae. While working on the phylogeny of the Ledellinae the presence of mitochondrial heteroplasmy was discovered.

Genetic variation was quantified within and among populations of deep-sea gastropod Benthonella tenella to identify the extent of population differentiation and potential mechanisms that might isolate gene pools. Benthonella tenella shows significant population differentiation among basins, especially between eastern and western corridors of the Atlantic. Genetic divergence among samples was correlated with depth on numerous scales - ocean-wide, within the western Atlantic, and within some basins.

Isolation by distance was not detected within the western Atlantic. Genetic population structure in this species is related to a complex array of evolutionary processes including depth-related environmental changes, historical events, topographic barriers and ocean currents

Most metazoan species have strict maternal inheritance of the mitochondrial genome. A unique inheritance pattern called, Double Uniparental Inheritance (DUI) occurs in at least seven bivalve families. In this system, males inherit and carry mtDNA from both parents, while females only carry mtDNA from the mother. Evidence of DUI was detected in two species of protobranch bivalves from the family Nuculanidae. Divergent 16S rRNA sequences were obtained within individuals for both Ledella ultima and Ledella sublevis. Ratios of homoplasmic to heteroplasmic individuals were approximately 1:1, in agreement with sex ratios in protobranchs.

The subfamily Ledellinae was used to test hypotheses about colonization and speciation patterns within the Atlantic. Phylogenies based on the mitochondrial 16S, and the nuclear 18S, 28S and H3 genes were developed. A monophyletic grouping of the Ledellinae was supported and consisted of Ledella ultima, Spinula species and other Ledella species. There is a bathymetric pattern of abyssal species Ledella ultima and the Spinula sp. forming a deep branch basal to the other confamilial species with a more recent radiation at bathyal depths.

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CHAPTER 1

INTRODUCTION

The deep sea covers more than two thirds of the earth's surface and supports a rich and highly endemic fauna (Hessler and Sanders 1967). This is surprising given the extreme conditions of perpetual darkness, high pressure, low temperature, and a meager food supply (Hessler and Sanders 1967). A variety of hypotheses have been proposed to account for this high diversity including: disturbance and predation (Dayton and Hessler 1972), niche diversification (Grassle and Sanders 1973; Etter and Grassle 1992), predation and productivity (Rex 1976), and the long stable history of the deep sea (Sanders 1968). Contemporary deep-sea research has focused largely on the ecological mechanisms that permit species to coexist. The evolutionary mechanisms that gave rise to this remarkable fauna are just beginning to be explored. The goal of my research is to reveal how evolution unfolded in this remote and immense ecosystem.

Most discussions of evolution in the deep-sea are general and speculative (Wilson and Hessler 1987; Gage and Tyler 1991) because the processes involved and the temporal, geographic, and bathymetric scales over which they operate are not well understood. In part, this is because molecular data, which are widely used to test

hypotheses about population differentiation and evolution in other environments, are difficult to obtain from the predominately formalin-fixed deep-sea samples. Techniques have been developed to extract and amplify DNA from small formalin-fixed specimens (Chase *et al.* 1998; France and Kocher 1996; Boyle *et al.* 2004). These techniques allow us to quantify geographic and bathymetric patterns of genetic variation and to test hypotheses about evolution in the deep-sea (outside of hydrothermal vents). The analysis of recent collections of frozen and ETOH preserved samples have improved knowledge of evolutionary processes in the deep sea; however, the geographic range of these samples is limited and differences in collection methods bias the taxonomic content. A combination of ETOH/Frozen samples and FFEP museum samples allowed me to develop species/family specific primers to phylogenetic hypotheses on broader geographic scales.

The focus of this dissertation is to explore several fundamental questions about the nature and scale of evolution in the deep sea by examining evolutionary processes on different levels. The first project was designed to investigate the scales of population differentiation and potentially speciation. Specifically, I examined whether population differentiation is associated with geographic, oceanographic, topographic or bathymetric factors. To address this, I quantified geographic and bathymetric patterns of genetic variation for an abundant and widely distributed caenogastropod, *Benthonella tenella* (Jeffreys). The second project tested hypotheses about how the deep Atlantic was colonized and determined the biogeographic patterns of a family-level radiation within the deep sea. To address this, I developed a geographically referenced phylogeny for the

diverse and broadly distributed protobranch bivalve subfamily, *Ledellinae.* In the process of working on the *Ledellinae,* I discovered evidence that this group has Doubly Uniparental Inheritance of their mitochondria. This type of mitochondrial inheritance is found in other bivalve families, such as the Unionidae and Mytilidae. This work is described in Chapter 3. I present it before the phylogeny chapter because the presence of mitochondrial heteroplasmy can complicate phylogenies based on mitochondrial genes.

In chapter two I explore the scales of population structure within a species. I quantified genetic variation within and among populations of the abundant and widely distributed deep-sea gastropod *Benthonella tenella* to identify the extent of population differentiation and potential mechanisms that might isolate gene pools. *B. tenella* is distributed throughout the North and South Atlantic with an unusually broad bathymetric range (500-5000m) that might be related to its planktotrophic larvae that presumably disperse in the surface currents. A fragment of the mitochondrial COI gene was amplified and sequenced in 113 individuals from museum samples that had been either formalin-fixed or dried upon collection. Twenty-four haplotypes were distributed among six ocean regions: the North American, West European, Argentine, and Guyana basins, the Gulf of Mexico, and the Mediterranean Sea. *Benthonella tenella* shows significant population differentiation among basins, especially between eastern and western corridors of the Atlantic. Genetic divergence among samples was correlated with depth on numerous scales - ocean-wide, within the western Atlantic corridor, and on smaller scales within some basins. Isolation by distance was not detected within the western

Atlantic. Within the North Atlantic there is isolation between the Eastern and Western corridors with a recent range expansion from the West European basin into the North American Basin. Overall, my results suggest that genetic population structure in this species is related to a complex array of evolutionary processes including depth-related environmental changes, historical events, topographic barriers and ocean currents. The strong genetic divergence between populations from the eastern and western corridors of the Atlantic might be sufficient to represent sibling species.

In chapter three I describe the detection of mitochondrial heteroplasmy in the deep-sea protobranch bivalve subfamily, Ledellinae. Most metazoan species have strict maternal inheritance of the mitochondrial genome. In bivalves, a unique inheritance pattern called, Double Uniparental Inheritance (DUI) occurs in at least seven bivalve families. In this system of mitochondrial inheritance, males inherit and carry mtDNA from both parents, while females only carry mtDNA from the mother. The evolutionary origins of DUI are unknown. Here I present evidence of DUI in two species of protobranch bivalves from the family Nuculanidae. Divergent 16S rRNA sequences were obtained within individuals for both *Ledella ultima* and *Ledella sublevis* with levels of divergence between putative male and female sequences of 40% and 15% respectively. Ratios of homoplasmic to heteroplasmic individuals were approximately 1:1, in agreement with sex ratios in protobranchs. This represents the first record of DUI in the protobranchs and suggests DUI evolved much earlier in the evolution of the Bivalvia than previously thought.

In chapter four, I present the phylogeny of the deep-sea bivalve subfamily Ledellinae. The mechanisms responsible for the origins of the diverse deep-sea benthos are not well known, and their evolutionary history is poorly resolved. This is especially true for the protobranch bivalves, which are more common in deep-sea habitats than in shallow water. I used geographically and bathymetrically referenced phylogenies to test hypotheses about the evolution of the endemic deep-sea protobranch bivalve subfamily Ledellinae (Family Nuculanidae). DNA from species in the genera *Ledella, Spinula, Bathyspinula*, and *Tindariopsis* was amplified from formalin-fixed ethanol preserved (FFEP), dried museum, frozen, and ethanol-preserved samples. The mitochondrial 16S and the nuclear 18S, H3 and 28S genes were amplified and sequenced using family and protobranch specific primers. Bayesian phylogenies based on 16S, 18S, and H3 indicate that the subfamily is monophyletic, but within the subfamily, genera are not. Some clades within the subfamily contain species from the Atlantic, Southern and Pacific Oceans, suggesting multiple colonization events among the oceans. The abyssal cosmopolitan species *Ledella ultima* appears to be one of the basal species in the subfamily. Some abyssal *Spinula* and *Bathyspinula* species are basal as well. A molecular clock suggests the *Ledella ultima* and species in the genera *Spinula/Bathyspinula* diverged much earlier than suspected anoxic periods around 90 and 65 mya. Thus some of these abyssal taxa are ancient suggesting they survived in deep refugia. A more recent radiation of species in the Ledellinae appears to have occurred after anoxic periods around 65 mya.

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CHAPTER 2

STRONG GENETIC DIVERGENCE WITH DEPTH AND GEOGRAPHIC SEPARATION IN THE DEEP-SEA GASTROPOD BENTHONELLA TENELLA (JEFFREYS)

Introduction

Numerous deep-sea organisms are thought to have remarkably large distributions that span entire oceans and in some cases may be globally distributed (Vinogradova 1979; Filatova and Shylenko 1992; Allen and Sanders 1997). If these morphologically species are defined accurately, we need to understand how such enormous ranges can be maintained. On one hand it may seem quite feasible because the deep sea is continuous with few obvious barriers to gene flow. However, most organisms are tiny (Thiel 1975), live in food-poor environments, have low fecundity and density (Rex et al. 2005; 2006), and release their offspring into extremely slow bottom currents. It is difficult to imagine how deep-sea species, even those with high dispersal potential, could maintain sufficient gene flow over such enormous geographic scales to retard divergence from either selective or nonselective forces.

We know little about the geographic scales of population structure in cosmopolitan deep-sea species because few studies have examined large-scale

(multibasin) patterns of genetic variation. Hydrothermal vent species have been the most intensively studied (e.g. Craddock et al. 1995; Vrijenhoek 1997; Won et al. 2003). Fewer multibasin studies have examined population structure in soft-sediment habitats, which cover most of the deep sea. The few studies exploring broad-scale patterns of genetic variation found considerable divergence at ocean-wide scales for brooding species or those with demersal larval dispersal (France and Kocher 1996; Weinberg et al. 2003; Zardus et al. 2006). No studies have examined genetic structure in a widespread soft-sediment species with planktotrophic larval dispersal from the deep sea.

Species with broad geographic ranges and high dispersal potential are generally expected to experience extensive gene flow and exhibit little population structure (Palumbi 1994; Bohonak 1999); however, few marine species fit this pattern (e.g. Garber et al. 2005; Castro et al. 2007). Broadly distributed species, including highly vagile pelagic fish (Graves 1998; Pogson et al. 2001; Áranson 2004), oceanic zooplankton (Bucklin et al. 1996; Goetze 2005) and transoceanic coastal species (Lessios et al. 2001) exhibit significant population structure, sometimes over small geographic scales (Reeb and Avise 1990; Barber et al. 2002; Palumbi 2004). Genetic structure within species with good dispersal ability reflects multiple factors that limit gene flow despite the potential for migration. Identifying barriers to gene flow is critical to understand how organisms evolve. While many putative isolating mechanisms have been identified in shallow-water systems, the mechanisms that structure the deep-sea fauna remain largely unknown (Etter et al. 2005; Zardus et al. 2006), but are likely to be similar to those in shallow ecosystems

(e.g. distance, historical factors, hydrodynamics) with the potential addition of novel or unique mechanisms (e.g. hydrostatic pressure, specific historical events).

Some deep-sea gastropods have planktotrophic larvae that are thought to undergo ontogenetic vertical migration to the surface waters (Bouchet and Warén 1979; 1994; Killingley and Rex 1985). If true, the dispersal potential of planktotrophic larvae may differ considerably from demersal nonfeeding larvae because they are likely to experience different levels of predation and starvation, and may disperse in surface or mid-water currents that differ in speed or direction from those near-bottom currents (Young et al. 1997). Planktotrophically developing snails have veliger larvae that feed and disperse in the plankton; lecithotrophic (non-planktotrophic) snails have a yolk sac (do not feed) and disperse by crawling away from the egg capsule after hatching or have a demersal swimming stage that is typically much shorter than planktotrophs (Bouchet and Warén 1994). Oceanographic currents can create barriers to gene flow that impact species differently depending on mode of development (Collin 2001). Historical changes in current paths can alter population connectivity (Muss et al. 2001; Barber et al. 2002) and oceanic fronts can isolate populations with planktonic dispersal (Sanjuan et al. 1996; Zane et al. 2000; Rogers et al. 2006). In the deep sea directional, historical or rate differences in currents at the surface, mid-water or deep water may have led to contrasts in population structure between planktotrophs and lecithotrophs.

Here I test several hypotheses about the nature and scale of population structure in *Benthonella tenella,* a common benthic deposit-feeding rissoid snail with planktotrophic development. *Benthonella tenella* is an important species that is widespread throughout

the Atlantic (Bouchet and Warén 1993) extends into the Pacific (Hasegawa 2005). It is one of the most abundant deep-sea snails, especially at abyssal depths (Rex and Etter 1990; Bouchet and Warén 1993; Flach and de Bruin 1999; Olabarria 2006). It has a large bathymetric range from 500-5000 m that varies in extent among ocean basins. Strong circumstantial evidence suggests that its larvae develop in the surface waters (Bouchet 1976; Bouchet and Warén 1979; Rex and Warén 1982; Killingley and Rex 1985).

Genetic variation of the mtDNA COI gene in *Benthonella tenella* from six ocean regions was quantified to identify the scales of geographic and bathymetric population structure and to test the relative importance of various topographic features (e.g. Mid-Atlantic Ridge), depth, and distance in affecting gene flow. Strong divergence occurred on several scales; between basins, across depth regimes, and between the Eastern and Western Atlantic. Although we detected a deep phylogenetic break between the Eastern and Western Atlantic, there is evidence of recent migrations from the East to West.

Methods

Formalin-fixed ethanol-preserved and dried specimens were used from previously collected samples in the following regions: the North American basin (NAB), West European basin (WEB), Guyana basin (GUY), Argentine basin (ARG), the Gulf of Mexico (GOM), and the Mediterranean Sea (MED). Three frozen samples from the Gulf of Mexico were also used (Table 2-1, Figure 2-1).

DNA was extracted using protocols described elsewhere (Chase et al. 1998a; Boyle et al. 2004). A 206 base-pair portion of the mitochondrial COI gene was amplified with the primers COIP3Bt2f and COIP3Bt2r (Boyle et al. 2004) in 50 µl reactions

consisting of 10 µl undiluted template DNA, 5 µl 10 X Thermophilic DNA polymerase buffer; 2.5 mM MgCl2; 20 *p*m each primer, 0.2 µM each dNTP, 2.5 units *Taq* (Promega), 0.5 µl Taqstart Antibody (Clontech) and H_20. The following Thermal Cycler protocol was used: 95˚C for 1 min; 5 cycles of 95ºC for 1 min, 56ºC for 1 min, and 72ºC for 1 min; 30 cycles 95º C for 30 s, 58 ºC for 30 s and 72ºC for 30 s. Both positive and negative controls (including an extraction negative control) were included. PCR products were visualized on a 1.5% agarose gel, and successful reactions were purified using a Qiaquick® PCR purification kit (Qiagen). Purified PCR products were sequenced in both directions with a *Taq* Big Dye kit (Applied Biosystems), ethanol precipitated, resuspended in formamide loading buffer and run on an Applied Biosystems model 373 Automated DNA sequencer (ABI), or on an ABI Avant 3100.

DNA sequences were edited and initially aligned in Sequencher 3.1 (Gene Codes Corp., Ann Arbor, Michigan). Alignments were confirmed using Clustal X (Thompson et al. 1997), then imported into MacClade 4 (Maddison and Maddison 2000) to facilitate haplotype identification.

A haplotype network was created using statistical parsimony (Templeton et al. 1992) in TCS 1.2 (Clement et al. 2000) and loops were broken using rules outlined in Pfenninger and Posada (2002). A haplotype tree was inferred using maximum likelihood in PhyML (Guindon et al. 2003) and HKY85 as a model for nucleotide substitution. Several outgroups were used to root the phylogeny: an unknown species from the genus *Benthonelliana* (GB XXXXX), *Cincinnatia winkleyi* AF118370, *Pyrgulopsis carinifera* AY627920 and *Littorina saxatilis* AJ132137.1.

To estimate the genetic diversity within each region, Arlequin ver. 2.000 (Scheinder et al. 2000) was used to calculate haplotype (*h)* and nucleotide (π*)* diversity for each basin. Samples from the Mediterranean were combined with samples from the West European basin for all analyses because all 4 of the individuals from the Mediterranean possessed haplotype M, the most common haplotype from the West European Basin.

Analysis of Molecular Variance AMOVA (Arlequin ver. 2.000; Excoffier et al. 1992) was used to test hypotheses about population structure at different geographic scales. To test for large-scale population structure among basins within the Atlantic, all stations more than two individuals, were including in an AMOVA test with samples grouped by basin.

The Mid-Atlantic Ridge (MAR) is an enormous mountain chain that might be an important topographical barrier to gene flow in the deep sea impeding the movement of larvae between the eastern and western Atlantic. To test the impact of this barrier on gene flow an AMOVA was conducted comparing samples on either side of the MAR. The eastern Atlantic mostly consisted of samples from the West European Basin and a few individuals from the Mediterranean; the Western corridor consisted of samples from the North American, Guyana, Argentine basins and the Gulf of Mexico. To detect structure within the western corridor, stations from the North American Basin, Gulf of Mexico, Guyana Basin and Argentine Basin were compared.

At smaller within-basin scales population structure was assessed within the West European Basin and within the North American Basin where sufficient sample sizes were

available. The North American Basin includes samples across a broad geographic and bathymetric range. To explore the affects of the more shallow southern samples from the deeper stations above 34 ˚N, samples were grouped based on depth and latitude.

Pair-wise genetic distances based on Φ_{ST} were used to construct trees depicting the relationships among stations (UPGMA) and among basins (neighbor joining) using PAUP* (Swofford 1998).

Patterns of genetic variation can be influenced by the geographic or bathymetric separation among samples. Partial Mantel tests were used to determine if genetic distance is related to the geographic distance or depth separating samples with the alternate independent variable held constant. Mantel tests were conducted using the R package (Casgrain and Legendre 2004). Negative Φ_{ST} values were converted to 0's and stations with less than two individuals were excluded. Relationships were examined at several geographic scales (entire Atlantic, Western Atlantic corridor, West vs. East Atlantic basins, and within the North American and West European basins).

Nested Clade Phylogeographic Analysis was used to test for associations between geographic patterns and haplotype distributions and to infer whether any associations are due to restricted gene flow or historical factors such as fragmentation, long distance colonization or range expansion (Templeton et al. 1995). A hierarchical series of nested clades were created from a haplotype network (Figure 2-2) by grouping haplotypes into 1 step, 2 step and 3 step clades following nesting rules in Templeton et al. (1987; 1992). The tip or interior status of each haplotype and clade was also characterized. For all nesting groups with more than one observed haplotype, a permutation analysis was

performed using Geodis 2.4 (Posada and Templeton 2000) to determine the clade distance D_c (a measure of the geographic spread of the clade) and the nested clade distance D_n (a measure of the distance of a clade from the geographic center of the higher clade which it is nested within). The average interior vs. tip (I-T) difference provides a contrast between old vs. young clades or haplotypes (Templeton 2004). Significant D_c, D_n or I-T values were interpreted with an inference key (http://darwin.uvigo.es/download/geodisKey_11Nov05.pdf). Net 2-P Kimura distances were used to estimate the genetic distances between the highest order clades (3 step).

Despite the controversy over NCPA (Knowles and Maddison 2002; Petit 2008; Knowles 2008; Templeton 2009), the analysis can be useful because unlike other methods it does not depend on defining populations by geography (Garrick et al. 2008; Templeton 2008; Felizola et al. 2008; Templeton 2009). This is important for deep-sea species because we have little basis to define population boundaries. The permutation analyses to test the significance of geographic and clade differences are statistically rigorous (Templeton 2009), but the inference key lacks any estimate of statistical error (Knowles and Maddison 2002; Petit 2008). Thus inferences based on the inference key must be interpreted with caution and, where possible, confirmed by other statistical analyses.

Isolation with migration model (IM, Hey and Nielson 2004) was used to test whether the North American and West European Basins have either had continuous gene flow or have been isolated with subsequent migration. The method estimates 6 parameters; effective population sizes of each population, rates of migration between the

two, the ancestral population size and time since population splitting between the North American Basin and West European basin. The following model parameters were used: HKY model, a mutation rate of 1.43 X 10^{-5} per gene per year (converted from COI molecular clock estimates of 1.83% per million years in Hydrobiidae; Wilke 2003), with a burn-in period of 10^6, 5 Metropolis chains, and repeated 9 times.

Levels of divergence for the fragment of COI amplified in *Benthonella tenella* were compared to divergence within and between closely related gastropod species. Sequences were downloaded from Genbank for the following species and subspecies: *Hydrobia glyca, H. ulvae, Oncomelania hupensis robertsoni, O. h. hupensis, Littorina littorea,* and *L. obtusata*. Species were selected for this analysis based on the availability of COI sequences from several populations and classification within the same superfamily as *Benthonella tenella* (Rissoacea). All sequences were trimmed by aligning them to the *Benthonella tenella* COI primers using Clustal X. Net 2-P Kimura distances were calculated within and between sister species using MEGA (Kumar et al. 2004).

Results

A 206 base-pair region of COI was sequenced (forward and reverse) from 113 individuals collected from 5 basins in the Atlantic Ocean and the Mediterranean. After trimming the primers, 158bp of the COI sequence were highly variable within and among basins with 24 unique haplotypes (sequences have been deposited in Genbank accession numbers XXXX-XXXX) (Table 2-2). There were 25 variable sites, 12 of which were parsimoniously informative; 20 were transitions, 6 were transversions and 8 were amino

acid changes. Haplotype and nucleotide diversity were highly variable among basins, with the NAB basin more diverse than the WEB (Table 2-3).

Sequences were amplified from samples collected by different collectors, at different times and preserved in different ways. The results were consistent across differences in sample preservation and time. For example, haplotype G was sequenced from FFEP samples collected during cruises 40 years apart, and from frozen samples collected recently. Haplotype M was sequenced from samples collected during 8 different cruises as part of different sampling programs, and either FFEP or dried. It was also recovered from samples that were collected over 100 years apart (for sample details see Boyle et al. 2004).

A parsimonious haplotype network indicated strong divergence among haplotypes from different ocean basins (Figure 2-2). The most abundant haplotypes A and M form distinct clades primarily found in the NAB or WEB respectively. Haplotype M differs from haplotype A by at least 7 nucleotide substitutions, indicating strong divergence between these clades. Although haplotype M and closely related haplotypes are most common in the Western European Basin, 4 individuals from shallower stations (800-1500 m) in the North American basin also possess haplotypes from this clade. Interestingly, the strong divergence between the NAB and WEB is associated with different depth regimes, NAB haplotypes are primarily from below 3500 m while those from the WEB are from above (Figure 2-3). Most haplotypes from the GOM and the Argentine and Guyana basins are closely associated with haplotype A, but there is a divergent group of Argentine basin haplotypes from below 3500 m (Figure 2-3). Along the Western corridor

of the Atlantic (NAB-GOM-Guyana-Argentine) there appears to be increasing haplotype divergence with distance from the NAB.

When stations were grouped based on genetic distance (UPGMA), they separate into two major clades by geographic origins with samples from the eastern Atlantic forming a separate clade from most of the western Atlantic samples (Figure 2-5). The shallow NAB stations S118, S2415, and S2664 grouped with the WEB clade, although S118 and S2415 remain quite distinct. Within the western Atlantic, samples from the deep NAB (>2886 m) formed a single clade while those from the GUY, GOM and ARG formed two clades associated with different depth regimes. The deepest station in the GOM, C12 (2924 m) is most closely related to the deep ARG basin stations S256 (3912 m) and S243 (3819 m). Shallower stations (546-2460 m) in the GOM, ARG, and GUY form a single clade.

Significant population structure exists within the Atlantic when samples were grouped by basin (Table 2-4 $p < 0.001$). The clear divergence between the eastern and western Atlantic haplotypes and samples (Figure 2.2) is consistent with the AMOVA, which indicates strong divergence across the Mid- Atlantic Ridge (Table 2-4 82.19% $p < 0.001$). Within the western Atlantic, population structure is weaker among basins (60.18%, $p = 0.06$) than within basin ($p < 0.001$) or within station ($p < 0.001$) (Table 2-4). The NAB and ARG basins differ genetically (Φ_{ST}) from each other and all other basins, whereas the GUY and GOM samples were similar (Φ_{ST})= 0.0903).

To determine if structure exists at within basin scales, AMOVA's were conducted within the WEB and NAB where sample sizes were larger. There was little structure

within the WEB, where most of the variation is within stations (92%, $p = 0.28$ Table 2-4). In contrast, significant population structure between stations ($p < 0.001$) emerges within the North American Basin (Table 2-4), primarily due to differences among stations from different depths. No structure is apparent if only the deeper samples North of 34° are included ($p = 0.14$, Table 2-4).

When samples are pooled within basins, an unrooted Neighbor-joining tree based on Φ_{ST} between basins (Figure 2-4), indicated that Guyana and the Gulf of Mexico were the most similar, and the Western Atlantic Basins are distinct from the West European Basin.

Mantel and partial Mantel tests were used to test whether genetic distances were correlated with the geographic distance or bathymetric difference among stations. At large scales (ocean wide and across the North Atlantic), genetic distance was correlated with both geographic distance and depth differences. However, at these scales geographic distance and depth are intercorrelated **(**$p<0.001$) making it difficult to resolve their relative importance (Table 2-5). Within the Western Atlantic (between NAB, GOM, GUY, and ARG) and within the WEB, genetic distances were influenced only by depth. For the NAB, genetic distance was correlated with both geographic and bathymetric separation, but the correlation broke down when either was statistically removed (Table 2-5).

Nested Clade Phylogeographic Analysis tests for significant associations between haplotype and geography and can be used to distinguish restricted gene flow from a variety of historical factors. The NCPA diagram is presented in Figure 2-6 and Table 2-5

outlines inferences based on significant results. Significant associations between geography and haplotype were found in clade 2-6, 3-2, and the total clade. Contiguous range expansion from the West European Basin to the North American Basin (interior to tip: clades 1-12 and 1-13) is inferred for clade 2-6 and the total clade. For clade 3-2 both 2 level clades (encompassing clade 2-4 North American Basin, and clade 2-3 North American, Gulf of Mexico, Guyana and 1 individual from the Argentine Basin) are internal (see Figure 2-6), thus the tip-interior status could not be determined.

The Net Average Kimura 2-parameter distance between clade 3-1 and 3-2 is within the range of distances observed within other gastropod species for this region of COI (Table 2-7, Table 2-8). However, the distance between clades 3-1 and 3-3 and 3-2 and 3-3 is greater, although still less than between species.

An Isolation with migration model produced the following parameter estimates: NAB population size $\theta_1 = 4N_1u = 16$, WEB population size $\theta_2 = 6.6$, Ancestral population size $\theta_A = 9.33$, population splitting time t= 8.175, migration from WEB to NAB m1= 0.295, migration from NAB to WEB m2= 0.005 (Figure 2-8, Table 2-9). The value for t should be interpreted with caution because the marginal posterior probabilities never settled down, but using the high point estimate time since splitting (t) can be very cautiously estimated at 566,000 generations. Despite much larger populations of *Benthonella tenella* in the WEB (Olabarria 2006), estimates of the effective population size in the NAB is greater than 2 times the estimates for the WEB. The migration rate from NAB to WEB is negligible and from WEB to NAB is 0.295 X generation time for

B. tenella, which is unknown. The nine replicate runs of the model produced consistent results with the Hi and Lo90 regions extensively overlapping.

A comparison of the net average Kimura 2-P distances within and between species for a fragment of COI indicates the net average for *Benthonella tenella* was 0.042, an order of magnitude higher than within species estimates for *Littorina littorea, L. obtusta*, and *Hydrobia glyca* (Table 2-8). However, the net average for *B. tenella* was less than between-species estimates and similar to the within species estimate of *Oncomelania hupensis robertsoni*.

Discussion

Despite its potential for long distance dispersal, the widely distributed benthic deep-sea snail *Benthonella tenella* exhibits strong population structure within the Atlantic Ocean. Populations differed on geographic and bathymetric scales, and appear to be influenced by the MAR and vicariance. The greatest genetic divergence occurred between Eastern and Western North Atlantic populations where there is evidence of past isolation with more recent migration from East to West. The population divergence across the North Atlantic exceeds the difference within the Western Atlantic corridor where samples ranged over much greater geographic distances.

Geographic Divergence between Eastern and Western Populations

The Mid-Atlantic Ridge bisects the Atlantic and represents the most obvious potential barrier to gene flow between the benthic environments of the eastern and western Atlantic. Populations of *Benthonella tenella* separated by the ridge exhibit

strong genetic divergence consistent with the notion that the MAR can impede larval transport of deep-water species. However, the larvae of *Benthonella tenella* are thought to disperse in surface currents (Killingley and Rex 1985; Bouchet and Warén 1994), raising the question of whether the divergence across the MAR reflects the ridge itself or the multitude of other potential explanations for divergence across such enormous scales (e.g. distance, selection, pressure, etc …)

Differentiation between Eastern and Western Atlantic Ocean populations has been documented mostly in species whose larvae would not be affected by the Mid-Atlantic Ridge such as shallow coastal amphi-Atlantic species (Wares and Cunningham 2001; Hickerson and Cunningham 2006), tropical species (*e.g.* the sea urchin, *Diadema sp.* Lessios et al. 2001) and an upper bathyal (200-1000 m) fish living mostly above the depth of the ridge (Abiom et al. 2005). One abyssal species that might be affected by the MAR, the protobranch bivalve *Ledella ultima*, exhibits modest divergence (Etter *et al.* 2011). In contrast, there is evidence of genetic population continuity for most other deep-sea species examined across the North Atlantic, (amphipods France and Kocher 1996; deep-water redfish Roques et al. 2002, crabs Weinberg et al. 2003, and bivalves Zardus et al. 2006). While this suggests the Mid-Atlantic ridge in general, is not an important barrier to gene flow for deep-sea species, relatively few species have been examined and in most cases sampling of stations and individuals was extremely limited. More individuals and stations were analyzed in both the West European and North American basins in this study than most of the previous studies, and unlike the majority of those previous studies we used a mitochondrial gene with greater resolution (COI

rather than 16s). However, *Benthonella tenella* is one of the few deep-sea species of those analyzed across this range that has vertically migrating planktotrophic larvae, which suggests the different type larval dispersal may result in differentiation across the Atlantic. *B. tenella's* larval disbursal may, in part, be influenced by forces that affect larval dispersal in shallow water species across these scales such as, distance, food availability, surface currents, and temperature. The contrast in population structure among deep-sea species also suggests there are differences in the factors that affect gene flow and large-scale population structure between species with planktotrophic or lecithotrophic larval dispersal.

The dispersal distances of deep-sea invertebrate larvae are generally unknown, although some deep-sea demersal lecithotrophic larva might disperse on larger scales than their shallow water counterparts due to lower metabolic rates (Lutz *et al.* 1986; Young 1994; Young et al. 1997). Young et al. (1997), hypothesized larvae from deep-sea planktotrophs have a lower chance of survival in surface waters due to greater predation, starvation or loss, which may limit their dispersal potential compared to lecithotrophs. Planktotrophic larvae also experience different currents, which vary in direction and speed with depth, from those experienced by lecithotrophic demersally dispersing larvae. Deep-sea species with planktotrophic larvae may be restricted to develop in more productive coastal waters, which may explain the greater differentiation between the Eastern and Western Atlantic than within the Western Atlantic over a longer geographic distance. These factors may increase the potential of population isolation in deep-sea planktotrophs compared to deep-sea lecithotrophs.

We may also consider that while there is evidence *Benthonella tenella* larvae spend time in surface currents or near surface currents, larval dispersal could also be affected by mid-water or bottom currents. Thus the Mid-Atlantic Ridge could be an important barrier to gene flow depending on the developmental stage or depth larvae are when they encounter the ridge. Based on known rates of shallow water larval dispersal, it is estimated it takes 9.25 days for swimming veligers to reach the surface from 4000m (Scheltema 1994), but it is unknown how long they spend at the surface. While rising to the surface and while sinking, mid- and deep-water currents might influence the direction of larvae dispersal. At the Mid-Atlantic ridge deeper currents tend to run north or south crossing over the ridge at several fracture zones, but these deeper currents can be weak and meandering (Bower et al. 2002; Lavender et al. 2005). Differentiation between the eastern and western populations suggests dispersal is infrequent across the MAR.

Other Geographic Patterns

There is no evidence of population divergence between the West European basin and the Mediterranean Sea (AMOVA $p > 0.05$). Mediterranean deep-sea populations are thought to be non-reproducing pseudopopulations (Bouchet and Taviani 1992). *Benthonella tenella* was considered an exception to this hypothesis because while other deep-sea species are rare it is quite common in the deep Mediterranean. The lack of divergence is surprising especially because of known barriers to gene flow in shallow water species (Dando and Southward 1981; Saavedra et. al 1990; Sanjaun et al 1996; Quesada et al. 1998; Zane et al. 2000; Baus et al. 2005). The shallow sill at the straight of

Gibraltar is often invoked as an isolating barrier, although some argue the Oran-Almeria front is responsible for isolation (Sanjuan et al. 1996; Zane et al. 2000). If the shallow sill is an isolating barrier for shallow water organisms it should be even more significant for deep-sea species with deep demersally dispersing larvae. Because *B. tenella* larvae are thought to disperse in the surface waters the sill should be less of a constraint than for most deep-sea species, but the Oran-Almeria front could be a barrier. The lack of divergence in *B. tenella* may reflect ongoing gene flow meditated by larvae dispersing in the surface currents, or a recent colonization of the Mediterranean as invoked for numerous shallow-water species (Patarnello et al. 2007).

Genetic distances were not related to geographic distance within the Western Atlantic Corridor over a range of 8,000 km. The lack of IBD may reflect strong northward surface currents that connect the Guyana Basin, the Gulf of Mexico and western North Atlantic (summarized in Shulman and Bermingham 1995) and suggests larvae can disperse considerable distances. There are internal haplotypes (Figure 2-7) found throughout the western corridor, which might indicate they are ancestral (Castelloe and Templeton 1994) which also explains the lack of measured IBD. These widely dispersed haplotypes found mostly bathyal depths also suggests the larvae of bathyal populations disperse more widely than abyssal populations, which may explain also the lack of isolation by distance as the bathymetric distribution of *B. tenella* also varies over the Western Atlantic. This implies gene flow between bathyal *B. tenella* populations is potentially greater than between abyssal populations, that dispersal of bathyal forms is

greater than abyssal forms, or that the bathyal environment is more conducive to retention of ancestral haplotypes.

Bathymetric Scales

The abundance and depth range of *Benthonella tenella* varies among basins and it is one of the few eurybathic species analyzed genetically (except for *E. gryllus*, France and Kocher 1996). Populations tend to be restricted to deeper abyssal ranges in the North American Basin above 34 ° N (2886-4970 m) with peak abundance between 3800-5000 m and in the Argentine basin (2460-5200 m). In contrast, in the eastern North Atlantic *B. tenella* is most abundant at upper bathyal depths between 1100-1400 m, but also has a broad depth range (400-4900 m) (Olabarria 2006). In the western Atlantic, below 34 °N, *B. tenella* has a narrow and shallow range e.g. 538 -2842 m in the North American, Guyana, and the Brazil Basins. However, the Guyana Basin is the only region below 34° N that was well sampled at deeper depths. It is also interesting to note that the few individuals that were available for this analysis from 2501-3500 m were scattered in their affinity with shallower or deeper populations (Figure 2-3). Strong population divergence across this depth range between 2500-3500 m suggests this might be an important region for population differentiation and speciation (France and Kocher 1996; Chase et al. 1998b; Etter et al. 2005; Zardus et al. 2006).

Population divergence is a function of both depth and distance separating samples at very large Amphi-Atlantic scales (Table 2-4), although the strong intercorrelation between depth and distance hampers our ability to partition relative importance. At

smaller within-basin scales or along the Western Atlantic corridor, genetic divergence appears to be primarily influenced by differences in depth. Along the Western Atlantic corridor abyssal populations of *B. tenella* from the North American and Argentine Basins are differentiated from each other and shallow Gulf of Mexico and Guyana Basin populations. However, the divergence is not as great as with depth in other deep-sea species on within basin scales (France and Kocher 1996; Chase et al. 1998b; Etter et al. 2005; Zardus et al. 2006). Also, unlike the bivalve *Deminucula atacellana,* the abyssal populations of *B. tenella* from the North American and Argentine Basins are more divergent from each other than from the shallower populations within the Western corridor.

It is unclear why the depth range of *Benthonella tenella* differs in the various Atlantic basins, but several spatial and temporal forces might conspire to control bathymetric range. For example, the populations above 34° N in the Western Atlantic may have shifted to abyssal depths during repeated periods of glaciation (Zachos *et al.* 2001), due to changes in bottom water; lower sea level, or more inhospitable conditions in the bathyal habitats during these periods. Diversity may be depressed during glacial periods as shown for other taxa due to changes in temperature, currents, or oxygen (Cronin and Raymo 1997; Yasuhara et al. 2008; 2009). Recovery of bathyal populations might have been hindered by changes in the species assemblage resulting in competition and predation from a diverse assemblage of gastropod species (Rex 1983). *B. tenella* is the most common and abundant gastropod at abyssal depths in the NAB (Rex *et al.* 1979; Rex and Etter 1990). Abyssal populations may have been more successful because fewer

species are adapted to abyssal habitats reducing competition or predation pressure. In the eastern North Atlantic (WEB), *B. tenella* is the most common and abundant gastropod in the upper bathyal zone (Flach and de Bruin 1999; Olabarria 2006), where diversity is depressed compared to the upper bathyal zone in the North American Basin (Stuart and Rex 2009). The success of *B. tenella* populations where diversity is low ecological interactions might play an important role in mediating bathymetric ranges.

Abyssal populations of *B. tenella* do not appear to be as continuously distributed as other abyssal species (Vinogradova 1976; Allen and Sanders 1997). In part, this could be due to poor abyssal sampling in some regions (Stuart *et al*. 2008). *B. tenella* is either absent or has very low abundance at abyssal depths in the West European and Guyana Basins compared to the North American and Argentine Basins. Abyssal species with demersal larvae might have more connected populations than species with vertically migrating planktotrophic larvae. The disjunct distributions may reflect the difficulties experienced by vertically migrating larvae. How do vertically migrating larvae from bathyal vs. abyssal populations return to an appropriate depth and habitat? Larvae may use habitat-specific cues to settle out as found in shallow-water species (reviewed in Kingsford et al. 2002). Perhaps, larvae from deeper populations are impacted by deeper currents, that larvae from shallower populations do not encounter.

Zoogeographic boundaries

Marine zoogeographic boundaries (Fischer 1960; Briggs 1974) are often set by the interaction between major currents, where water mass and its characteristics (especially temperature) change (Fischer 1960). Changes in flow can also set range

limits for species (Gaylord and Gaines 2000; Collin 2001; Wares et al. 2001). *Benthonella tenella* differs genetically across some of these known biogeographic boundaries. The NAB populations of *B. tenella* diverge at Cape Hatteras 34° N and Cape Lookout 32° N where a strong zoogeographic boundary exists for both shallow-water (Briggs 1974) and several deep-water taxa (Cutler 1975; Hilbig 1994). The zoogeographic break between 35-40°S (Briggs 1974) corresponds to a separate clade of haplotypes of *B. tenella* in the Argentine basin. Both of these breaks also correspond to shifts in bathymetric range. This implies factors that create zoogeographic boundaries in shallow and deep-water taxa also correspond to change in population structure of *Benthonella tenella*. Because *B. tenella's* larvae travel in currents the change in flow across zoogeographic boundaries may impact its population structure.

Historical factors

The nested clade analysis suggests the population structure within the North Atlantic reflects historical factors. Geographic patterns of genetic variation for clade 2-6 (West European Basin) and the total clade suggested contiguous range expansion. The genetic signature of range expansion is based on known range expansion events on land after events such as glaciation (e.g. Templeton 2004) and is characterized by older internal haplotypes geographically restricted while younger tip haplotypes are either widespread geographically or distantly located from the interior (Templeton et al. 1995). Contiguous range expansion is thought to result from "individual short distance dispersal" (Templeton et al. 1995). For the WEB clade, most of the shallow NAB

individuals possess tip haplotypes indicating the direction of range expansion is from East to West.

The IM analysis supports migration from East to West after isolation, but not from NAB to WEB. For shallow-water cold temperate species, the spread of species across the Atlantic also is thought to be from East to West (Vermeji 1991; 2005) and is associated with recolonization of western Atlantic habitats after glaciation events. In the deep, the migration from East to West may represent a combination of factors. Very large populations of *B. tenella* in the East (Flach and de Bruin 1999; Olabarria 2006) could produce enough larvae that by chance more survive transatlantic migration than from NAB populations. The WEB is closer to the Mid Atlantic Ridge thus larvae from the East may cross it in surface currents before sinking. The WEB populations are mostly bathyal populations thus their larvae may reach the surface in less time than larvae from the mostly abyssal NAB populations, which may lead to them dispersing longer distances in faster surface currents than abyssal larvae which may be more impacted by slower deep-/mid-water currents that may be moving in a different direction than surface currents.

Genetic differences between the East and West suggest the populations have been isolated for a long time. Using phylogenetic analyses we cautiously interpret that Eastern populations were derived from Western populations (Figures 2-2 and 2-4). The divide between east and west may be the result of a past historical event isolating the populations for a long time and maintained by some of the factors mentioned above. Using IM analysis, we could not fully resolve time of splitting as the posterior probability

range was very broad, but using the high point as a rough estimate of time since splitting we estimate 566,000 generations. Assuming one-year generation time, common for many shallow water gastropods, this translates to 566,000 years. However, generation time may be much longer for deep-sea organisms, potentially even 10 years, which suggests a much longer estimate of 5.6 mya years. Given both the uncertainty in the IM estimate and in generation time, more genetic markers are needed to more accurately resolve the splitting time estimate (Nielson and Wakeley 2001), and potentially infer generation time (Fu 2001; Drummond et al. 2003) in order to postulate a specific historical cause of the populations splitting. However, the time range overlaps with the period estimated for population divergence in a bathyal fish across a similar spatial scale and was associated with the mid Pleistocene transition (approximately 1.2 Ma to 0.6 Ma) (Abiom *et al.* 2005). Over this very broad time period many climate changes occurred potentially isolating these populations. For example, changes in current patterns associated with the closing of the Isthmus of Panama (Muss *et al.* 2001), drops in sea level with multiple ice ages (Zachos *et al.* 2001) which might have made the ridge a more prominent barrier, or disruption to global deep-water circulation (Kawagata et al. 2005).

Species status?

Benthonella tenella populations between the Eastern and most Western stations were highly divergent genetically and morphologically. Bouchet and Warén (1993) argued that the morphological variation described by others as several different species (*Benthonella tenella, B. gaza, B. fischerii*) represents a single polytypic species. We

found that morphological variants were not associated strictly with genetic divergence. In general, individuals with the same phenotype do not necessarily share similar haplotypes - e.g. all the specimens from the North American Basin deep and shallow were similar morphologically (*B. gaza* type), but differed genetically, while individuals from the Gulf of Mexico varied phenotypically (*B. gaza* and *B. fischerii* types), but were similar genetically. Morphotypes vary in degree of sculpture and over-all shape. Similar morphological variation (sculptured vs. smooth) is known from another species of rissoacean gastropod and was attributed to environmental plasticity (Davis et al. 2006). The amount of genetic variation observed between eastern and western populations may be indicative of a cryptic species, but this should be determined from multiple lines of evidence (Wiens and Penkrot 2002; Sites and Marshall 2004; Puilandre *et al.* 2009).

We compared the region of COI amplified by the *Benthonella* primers with publish sequences for caenogastropod species for which there were multiple haplotypes within species including *Littorina littorea* and *L. obtusata* and other gastropods in the same superfamily as *B. tenella*- Rissooidiea (Table 2-8). Species in the genera *Littorina* and *Hydrobia* have lower within species distances than *B. tenella*. However, in another Rissooidea species (*Oncomelania hupensis robertsoni*) there was a higher level of within-species divergence. Few gastropods have been genetically analyzed at oceanic scales, making it difficult to determine if the level of variation observed is reflective of species status. Also it is difficult to determine species status by a small fragment of one gene. The West European Basin populations may be a separate species or in the process of

speciation, but the Eastern and Western Atlantic populations have probably been separated for a long time

Conclusions

Populations of the widely distributed deep-sea species *Benthonella tenella* are not panmictic throughout the Atlantic. The lack of IBD over large regions of the Atlantic suggests dispersal is effective across vast distances, but various contemporary and historical factors may impede gene flow and create divergence among basins. Between the Eastern and Western corridors of the Atlantic isolation was probably caused by historical event(s). The apparent discontinuous distribution of abyssal populations in the North American and Argentine basins may be indicative of separate invasions of abyssal depths. Genetic divergence among basins was also correlated with bathymetric range shifts suggesting depth may play an important role in population differentiation at basin wide scales. The Mid-Atlantic Ridge may also affect gene flow among eastern and western Atlantic populations of *Benthonella tenella* and may reflect its unusual mode of larval development.

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FIGURE 2-1: Map of stations. Colored dots represent the area where stations occur: Green= West European Basin and Mediterranean, Red= North American Basin, Blue=Gulf of Mexico, Purple=Guyana Basin and Yellow= Argentine Basin.

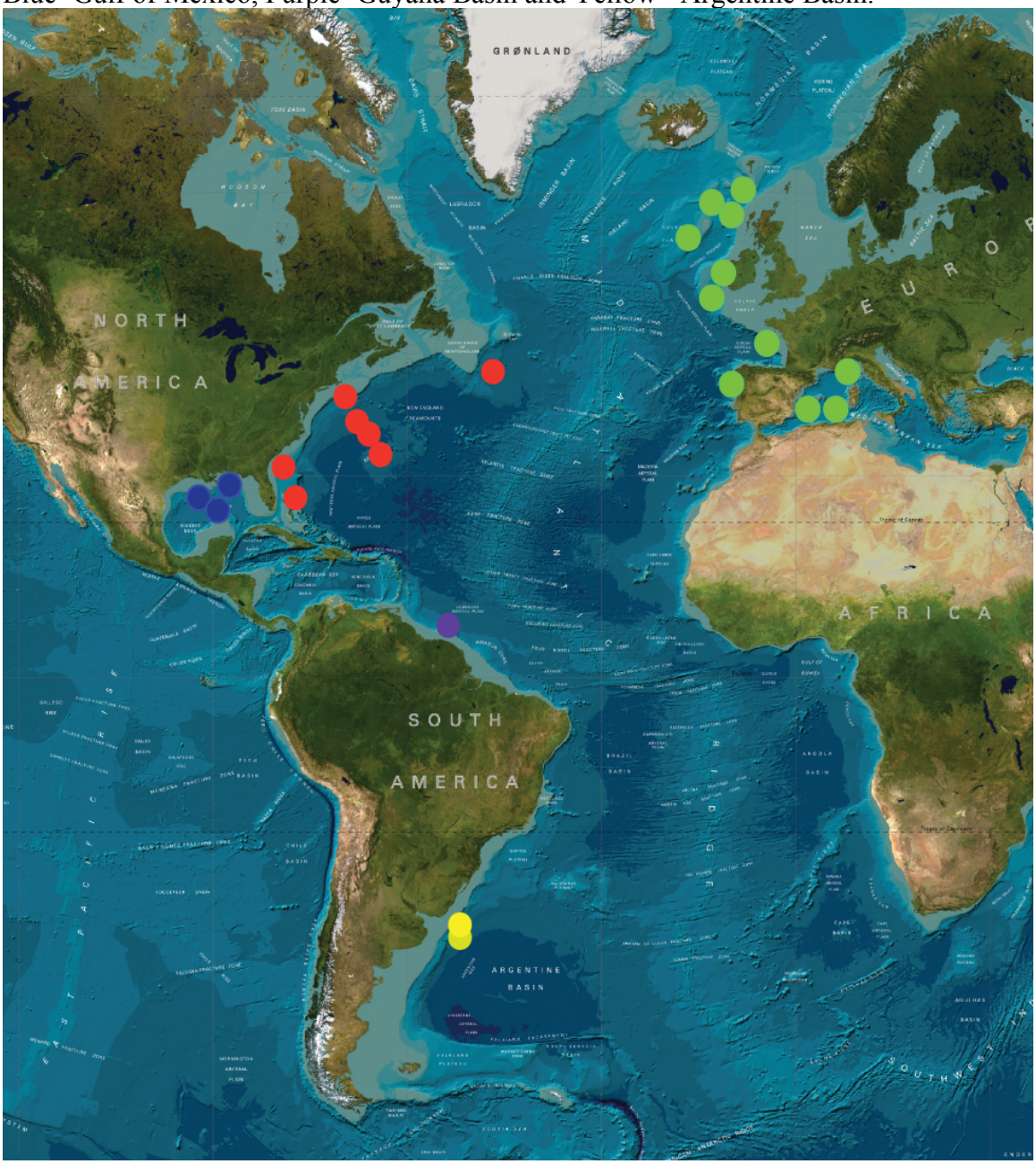

TABLE 2-1: *Benthonella tenella*, station numbers, ocean region, depth, latitude, longitude and source of samples.

Region	Station	Depth	Latitude	Longitude	Source
Argentine	262	2460	36 05'S	52 17'W	WHOI
	243	3819	37 36'S	52 23'W	WHOI
	256	3912	37 40'S	52 19'W	WHOI
Gulf of Mexico	BH	546	27 28'N	91 16'W	DGoMB
	S36	1825	28 55'N	87 40'W	DGoMB
	C12	2924	26 13'N	89 08'W	DGoMB
Guyana	295	1487	8 04'N	54 21'W	WHOI
North American	2668	538	30 58'N	79 38'W	Albatross
	2415	805	30 44'N	79 26'W	Albatross
	118	1144	32 19'N	64 34' W	WHOI
	2654	1207	27 57'N	77 27'W	Albatross
	64	2886	38 46'N	70 6'W	WHOI
	77	3806	38 00'N	69 16'W	WHOI
	85	3834	37 59'N	69 26'W	WHOI
	334	4400	40 42'N	46 14 'W	WHOI
	70	4680	36 23'N	67 58'W	WHOI
	84	4749	36 24'N	67 56'W	WHOI
	93	4967	34 39'N	66 26'W	WHOI
	80	4970	34 49'N	66 34'W	WHOI
West European	ES73no4	900	60 10'N	8 12'W	SAM
	J40	946	49 01'N	12 05'W	Porcupine
	J39	1019	49 01'N	11 56'W	Porcupine
	J41	1068	49 04'N	12 22'W	Porcupine
	313	1496	51 32'N	12 35'W	WHOI
	ES252	1510	58 52'N	12 53'W	SAM
	ES255	1595	58 26'N	12 42'W	SAM
	ES257	1700	57 55'N	12 18'W	SAM
	44	1739	43 40'N	3 35'W	Sarsia
	J16	1818	39 55'N	9 56'W	Porcupine
	ES112	1900	55 12'N	15 50'W	SAM
	9753	1942	50 54'N	12 10'W	Discovery
	DS01	2091	57 59'N	10 40'W	Incal
	ES176	2245	57 15'N	10 26'W	SAM
Mediterranean	J51	2588	36 55'N	1 10'E	Porcupine
	J54	2758	37 41'N	6 27'E	Porcupine
	217	2775	41 05'N	7 25'E	WHOI

TABLE 2-2: Haplotype frequency per station and basin. Depth is in meters, n is the number of sequences per station.

HAPLOTYPE FREQUENCY

Station	Depth(m)	n	A	B	C	D	F	G	H	I	K	L	LL	M	N	O	P	Q	R	S	T	U	V	W	X	Y
Argentine																										
262	2460	1							1																	
243	3819	1									1															
256	3912	4										1	1													2
Gulf of Mexico																										
BH	546	1																				1				
S36	1825	4						3	1																	
C12	2924	1																					1			
Guyana																										
295	1487	3			1			1	1																	
North American																										
2668	538	1								1																
2415	805	1																1								
118	1144	1																			1					
2654	1207	2												1		1										
64	2886	1	1																							
77	3806	9	6			2		1																		
85	3834	8	7	1																						
334	4400	2	1		1																					
70	4680	5	5																							
84	4749	7	7																							
93	4967	6	5				1																			
80	4970	7	7																							
West European																										
ES73	900	1												1												
J40	946	1															1									
J39	1019	1																1								
J41	1068	2												2												
313	1496	4												4												
ES252	1510	3												2											1	
ES255	1595	1												1												
ES257	1700	2												2												
44	1739	1												1												
J16	1818	2												1	1											
ES112	1900	2												2												
9753	1942	18												17						1						
DS01	2091	1												1												
ES176	2245	5												4												1
Mediterranean																										
J51	2588	1												1												
J54	2758	2												2												
217	2775	2												2												

TABLE 2-3: Average haplotype (*h*) and nucleotide diversity (**π**) per Basin and per stations with n>2, n=the number of individuals. Basin value includes all individuals sampled.

Basin	**n**	***h***	**π**
Argentine	6	0.933 ± 0.122	0.025 ± 0.017
256	4	0.833 ± 0.2224	0.0158 ± 0.013
North American	48	0.409 ± 0.091	0.011 ± 0.007
77	9	0.556 ± 0.165	0.005 ± 0.005
85	8	0.333 ± 0.215	0.002 ± 0.003
70	5	0.00±0.00	0.00±0.00
84	7	0.00±0.00	0.00±0.00
93	6	0.333 ± 0.215	0.002 ± 0.003
80	7	0.00 ± 0.00	0.00±0.00
Guyana (295)	3	1.000 ± 0.272	0.008 ± 0.009
West European /Mediterranean	44	0.257 ± 0.087	0.002 ± 0.002
313	4	0.00±0.00	0.00±0.00
ES252	3	0.667 ± 0.314	0.004 ± 0.005
9753	18	0.111 ± 0.096	0.001 ± 0.001
ES176	5	0.400 ± 0.237	0.003 ± 0.003
Gulf of Mexico	6	0.800 ± 0.172	0.008 ± 0.007
S36	4	0.500 ± 0.265	0.003 ± 0.004

FIGURE 2-2: COI haplotype network. Lines represent one mutational step. The area of the circle is proportional to the number of individuals with that haplotype. The color of the circle represents the location where that haplotype was found. Small-unfilled circles represent hypothetical intermediate haplotypes. Asterisks indicate that the haplotype corresponds to an amino acid change from Haplotype A.

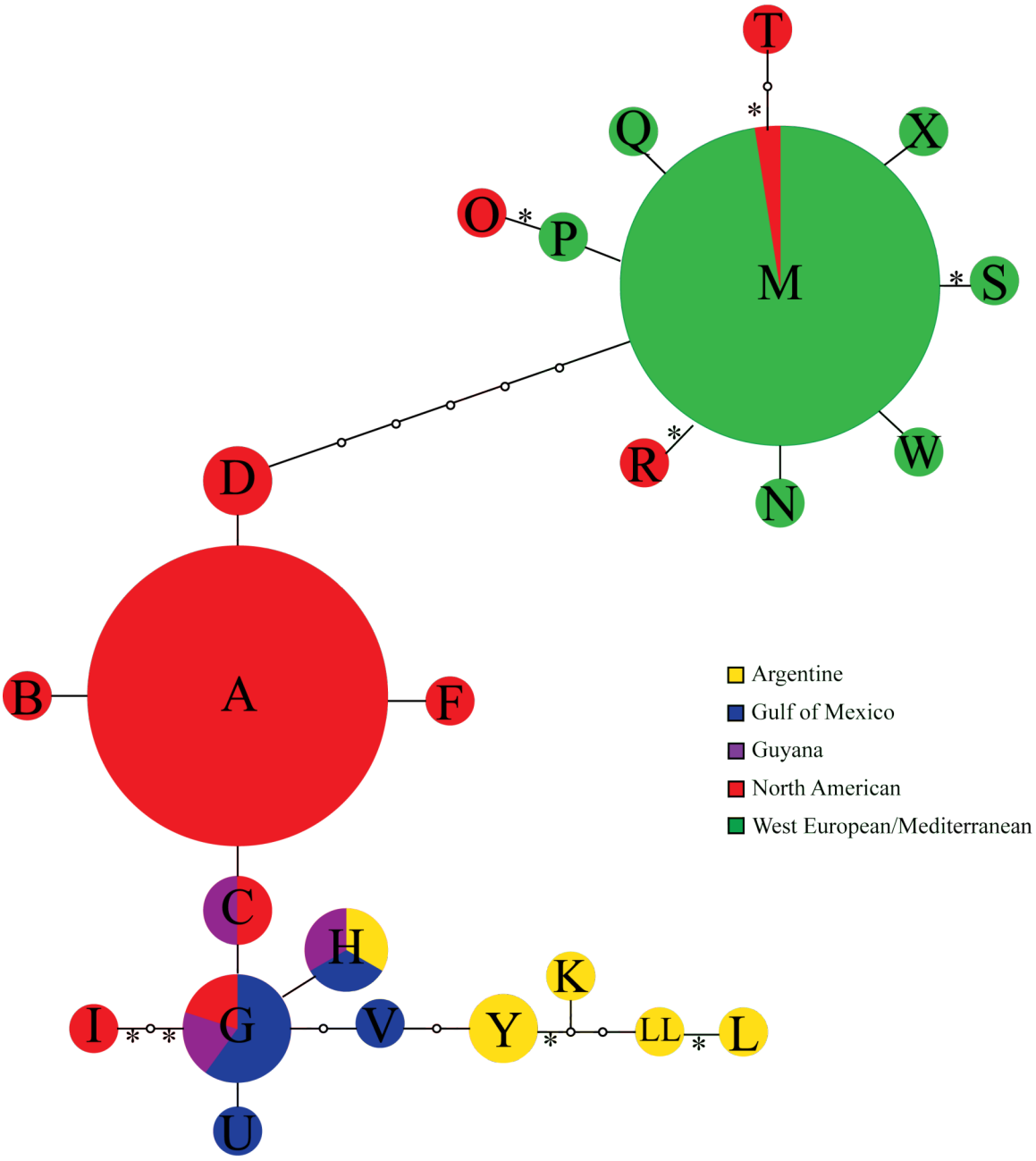

FIGURE 2-3: COI Haplotype network. Circle color indicates depth. Area of the circle is proportional to the number of individuals with that haplotype.

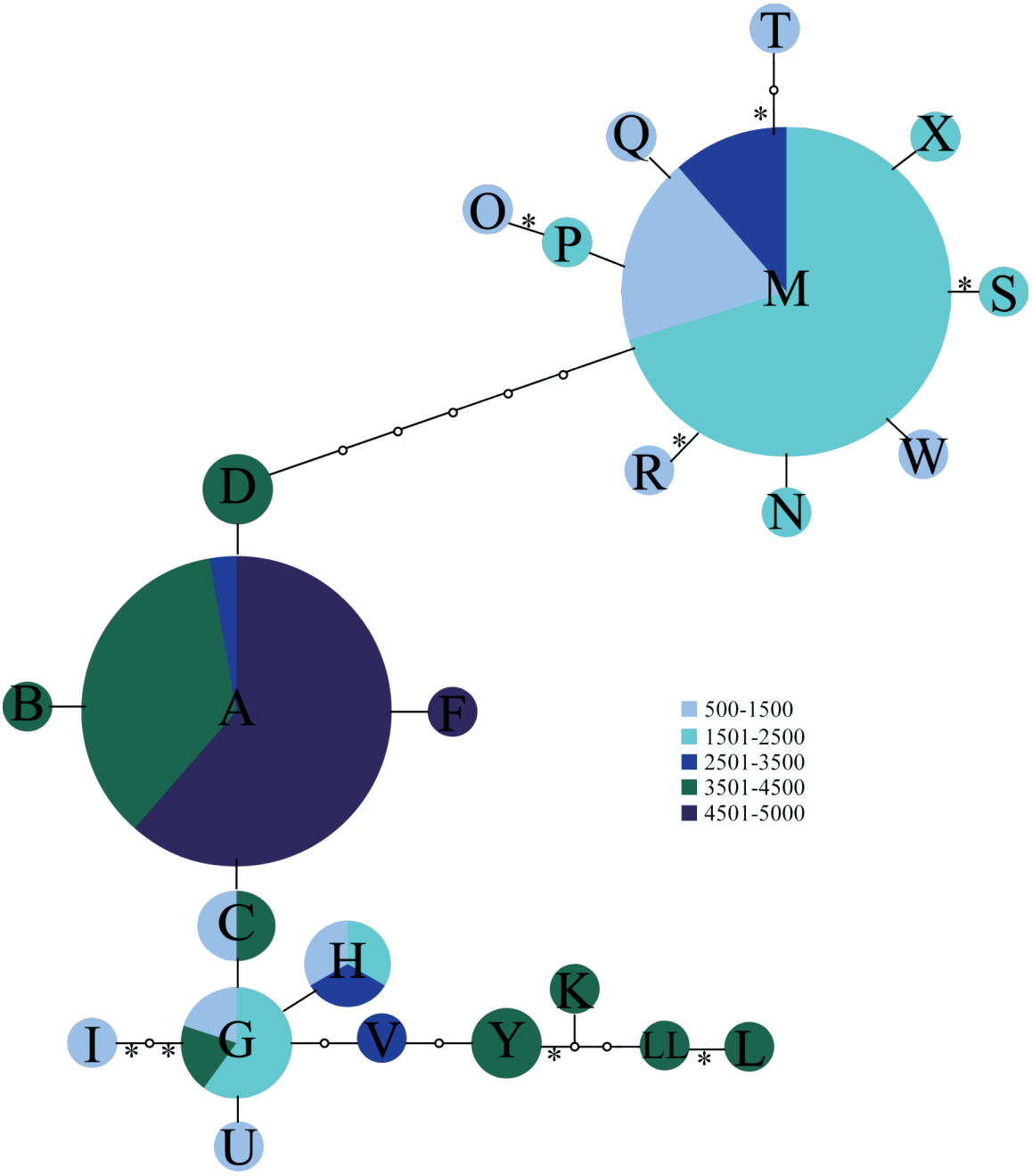

FIGURE 2-4: Maximum likelihood tree of *Benthonella tenella* COI haplotypes rooted with several outgroups. Bootstrap values greater than 50 are indicated on branch.

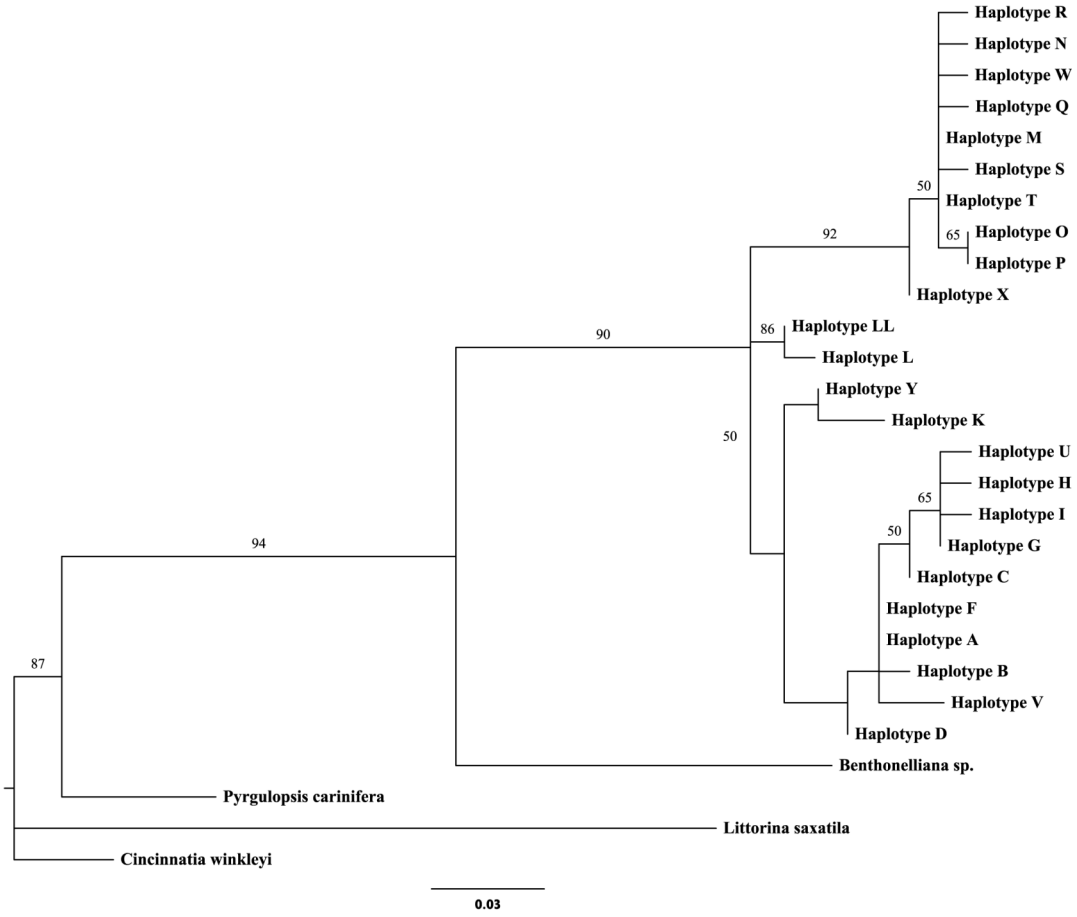

FIGURE 2-5: UPGMA distance tree of stations based on ΦST distances.

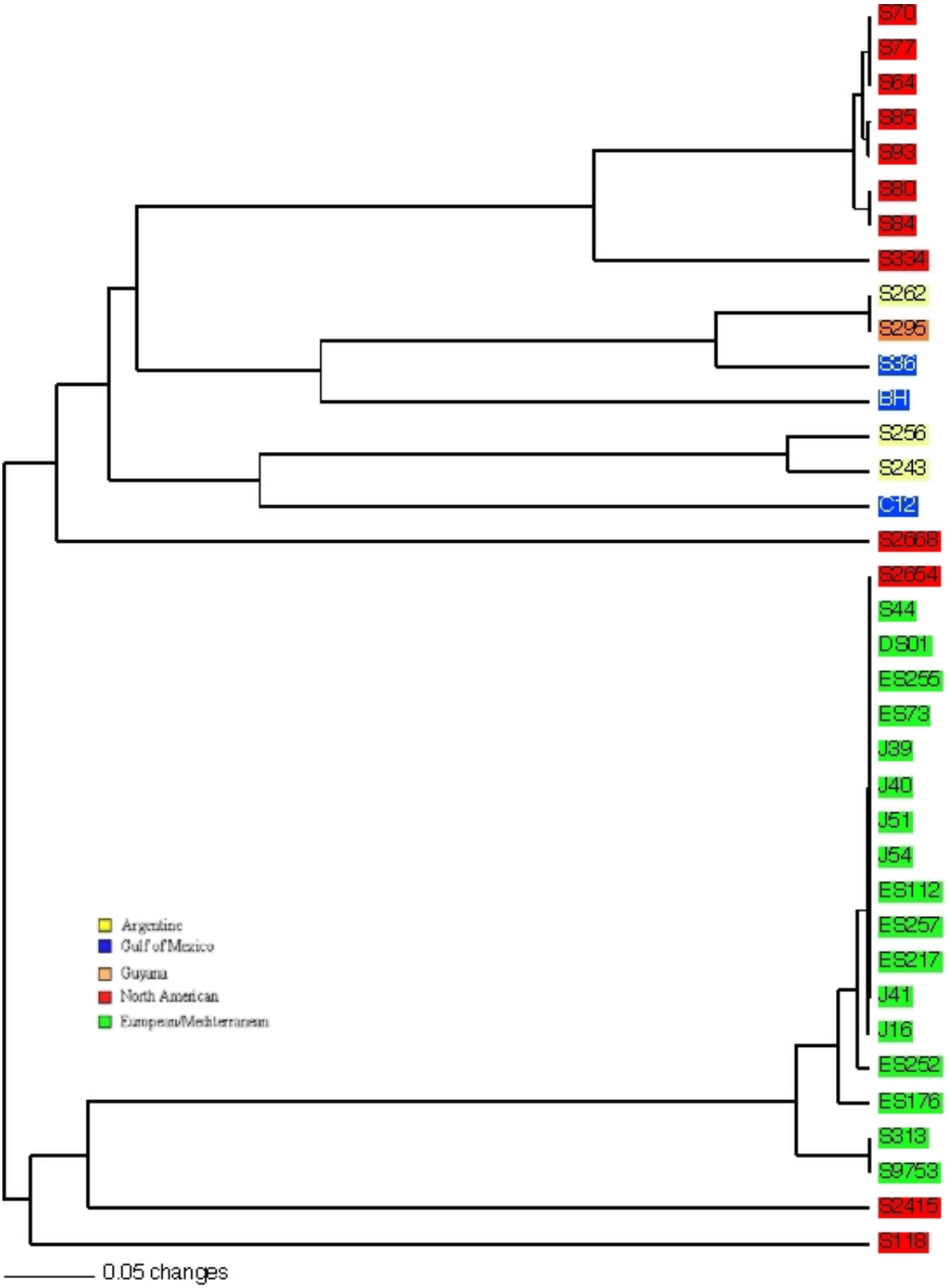

TABLE 2-4: AMOVA results testing for population structure with and among basins and regions. Kimura2P model was used to calculate ΦST.

AMOVA	Source of variation	d.f.	Sum of squares	Variance component	% of total	P value
All Basins	Among Basins	4	172.517	V(A) 2.92	**87.67**	**<0.0001**
	Among stations w/in basin	15	17.006	V(B) .205	**6.17**	**<0.0001**
	W/in station	75	15.397	V(C) .205	**6.16**	**<0.0001**
	Total	94	204.92	3.334		
EAST vs. West	Between East and West	1	144.408	V(A) 3.01	**82.19**	**<0.0001**
	Among stations w/in Basin	18	39.792	V(B) 0.452	**12.33**	**<0.0001**
	W/in Basins	76	15.268	V(C)0.201	**5.49**	**<0.0001**
	Total	95	199.469			
Within West	Among Basins	3	23.335	V(A).964	60.18	0.06
	Among stations w/in basin	7	15.386	V(B).357	**22.32**	**<0.0001**
	W/in station	44	12.333	V(C).280	**17.50**	**<0.0001**
	Total	54	51.055	1.60		
Within West European	Among stations	8	.989	V(A).0079	7.74	0.26
	Within stations	31	2.911	V(B) .0939	92.26	
	Total	39	3.9	0.102		
Within North American	Among stations	7	15.387	V(A)0.369	**67.64**	**<0.0001**
	Within stations	37	6.524	V(B)0.176	**32.36**	**<0.0001**
	Total	44	21.911	.545		
W/in NAB Above 34°N	Among stations	6	1.220	V(A)0.008	5.12	0.14
	Within stations	36	5.524	V(B)0.1533	94.88	
	Total	42	6.744	0.161		

FIGURE 2-6: Neighbor joining tree of pair-wise distances between basins based on Φst values.

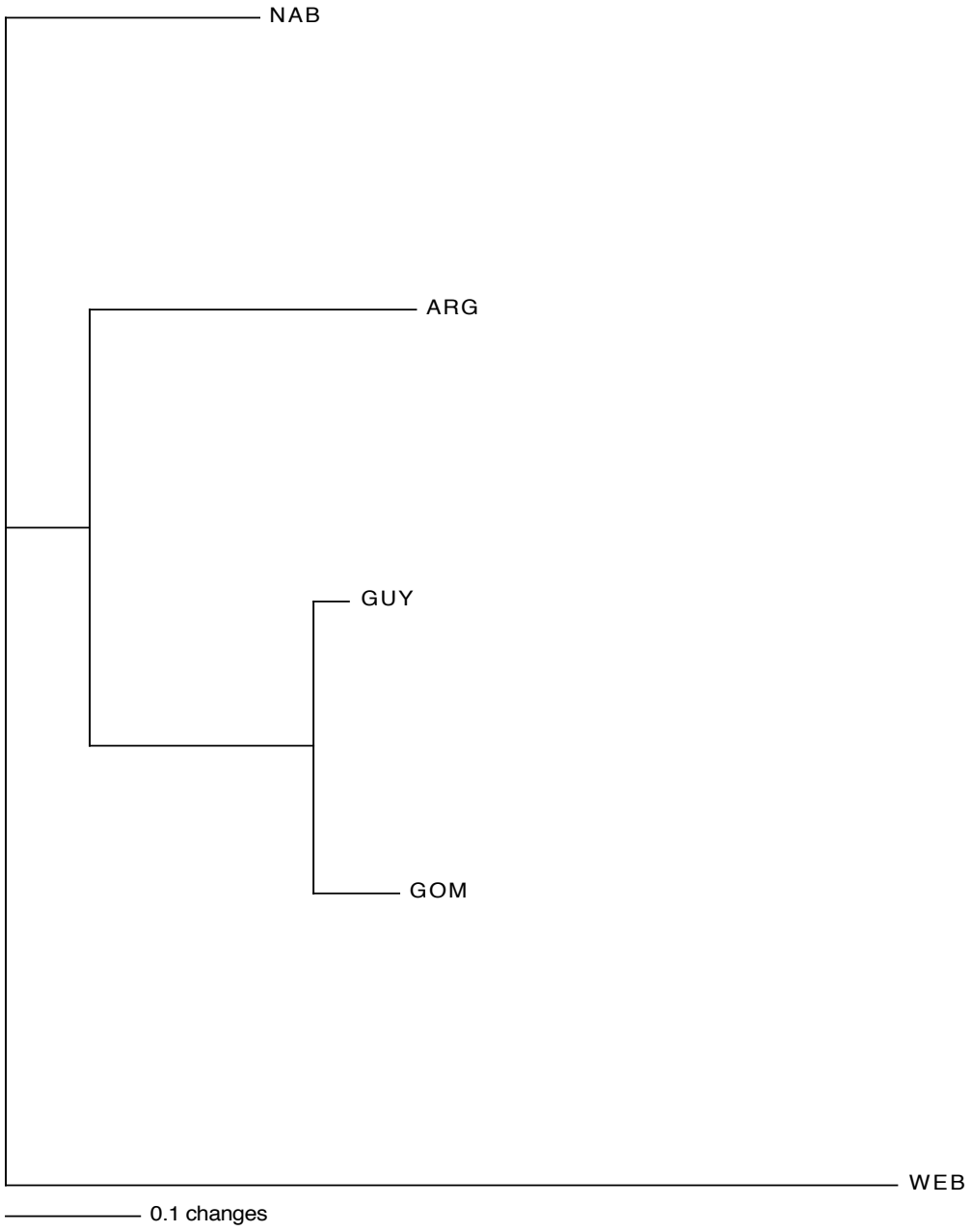

TABLE 2-5: Mantel tests for whether Genetic distance as measured by ΦST is correlated with geographic distance (km) or depth (m); and partial Mantel tests where geographic distance or depth was removed. R values are Spearman's R. N is the number of stations (populations) in the analysis. Stations with single individuals were excluded. 9999 permutations were run.

Genetic Distance vs.:	All Basins (N=21)		NAB (N=8)		WEB (N=10)		WEBvsNAB (N=18)		Western Atlantic (N=11)	
	R	p	R	p	R	p	R	p	R	p
Geog.	0.515	**< 0.001***	0.60	**0.048**	-0.035	0.361	0.619	**< 0.001***	0.407	0.057
Depth	0. 591	**< 0.001***	0.580	**0.048**	-0.359	**.0455**	0.769	**< 0.001***	0.627	**< 0.001***
Geog. w/o Depth	0.510	**< 0.001***	0.490	0.13	0.166	0.191	0.410	**< 0.001***	0.355	0.09
Depth w/o Geog.	0.587	**< 0.001***	0.456	0.089	-0.389	**0.027**	0.671	**< 0.001***	0.603	**0.001***

FIGURE 2-7: Nested clade diagram.

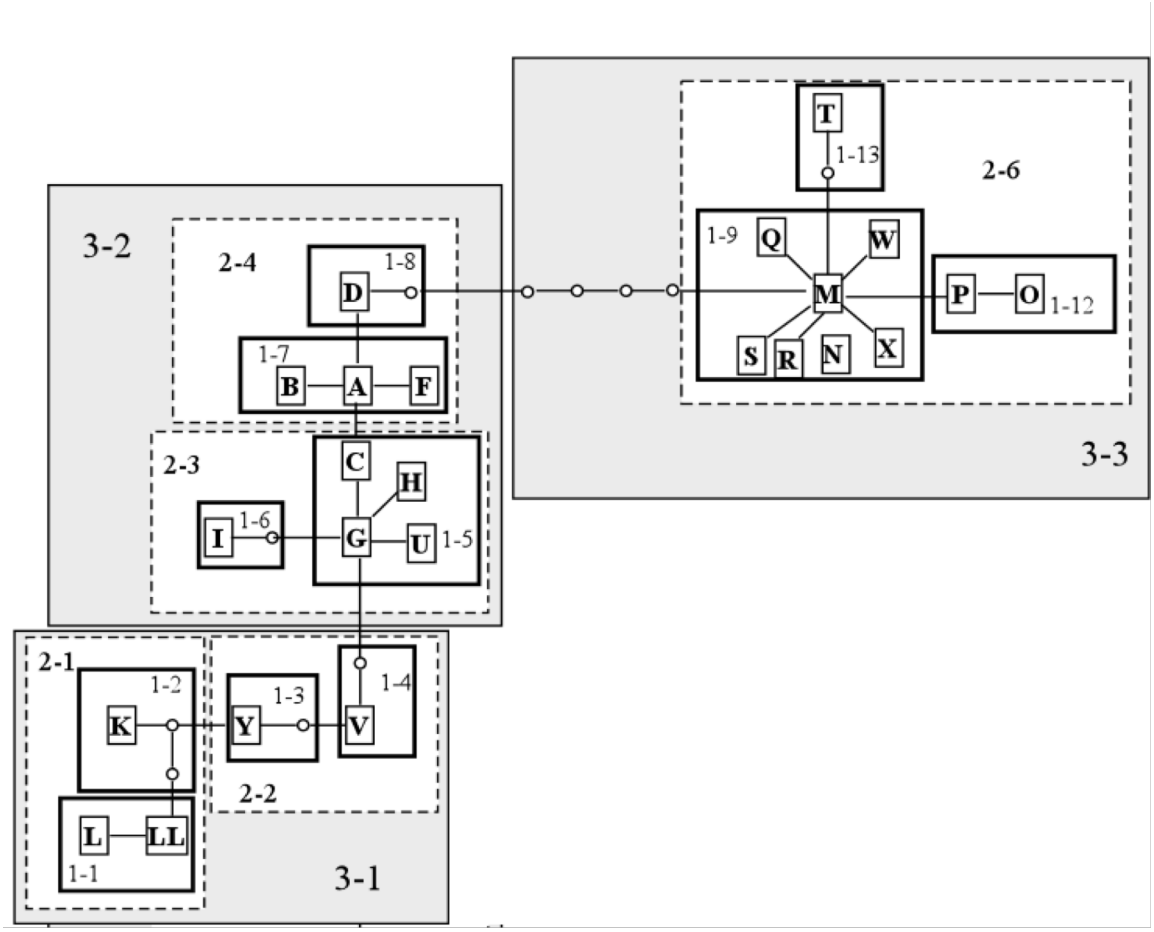

TABLE 2-6: Inference chain for the results of the Nested Clade analysis for *Benthonella tenella.*

Nested Clade	**Chain of inference**	**Inferred outcome**
Total Clade	1,2,11,12 no	Contiguous range expansion
3-2	1,2-	inconclusive (no tips)
2-6	1,2,11,12 no	Contiguous range expansion

TABLE 2-7: Net Average 2 Kimura P distance between 3 step clades.

Clade		
3-1	3-1	3-2
3-2	.017	
3-3	.039	.048

FIGURE 2-8: Posterior probability densities for the isolation with migration (IM) model A. Effective population size θ1=North American basin, θ2= West European Basin B. Migration rates m1=migration from WEB to NAB, m2=migration from NAB to WEB, C. Estimate of ancestral θ and D. Time since most recent common ancestor/mutation rate.

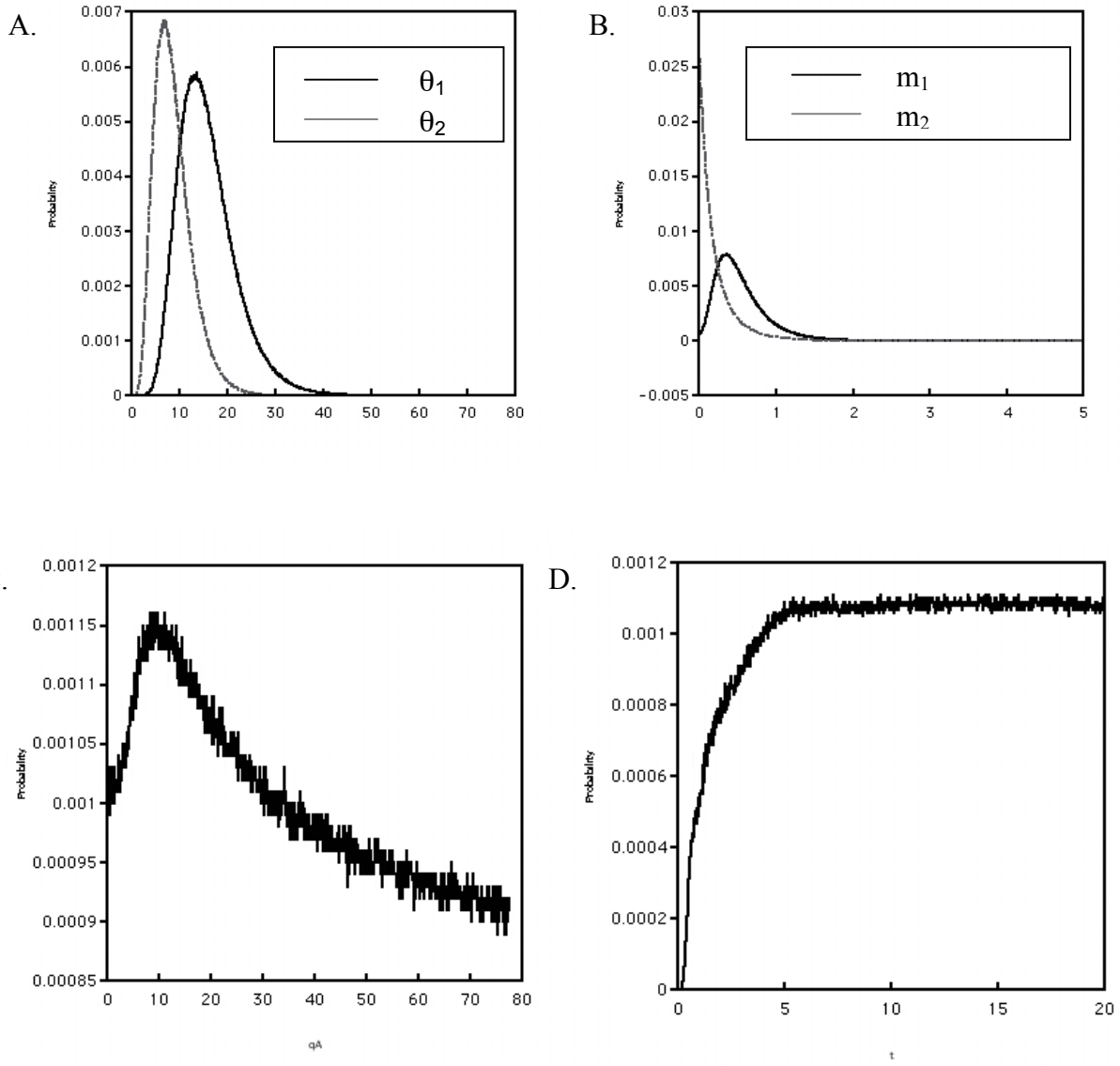

TABLE 2-8: Average K2P distance within and net distance between species of selected caenogastropods for COI section amplified by *B. tenella* primers: *Rissooidea species.

Species	Distance within species	Distance between species	# of haplotypes
**Benthonella tenella*	0.042		24
**Hydrobia glyca*	0.005		44
**Hydrobia ulvae*	0.012	0.1	18
Littorina littorea	0.002		93
Littorina obtusata	0.004	0.115	47
**Oncomelania hupensis robertsoni*	0.040		61
**Oncomelania h. hupensis*	0.019	0.063	137

TABLE 2-9: Population and migration parameters estimated from IM analysis.

Population size	Estimate	Lo90	Hi90	N_e	Lo90	Hi90
NAB θ_1	16	8.74	28.37	279,720	152,692	495,883
WEB θ_2	6.6	2.60	14.27	115,384	45,455	249,510
Ancestral θ_A	9.33	0.08	159.50	163,112	1398	2,788,461
Migration				**Ind/gen**		
WEB to NAB m_1	0.295	0.06	0.795	2.36		
NAB to WEB m_2	0.005	0.005	0.515	0.017		
Timing				**Years**		
splitting time t	8.175	6.28	49.97	566,000	439,160	3,493,007

CHAPTER 3

EVIDENCE FOR AN ANCIENT ORIGIN OF DOUBLE UNIPARENTAL INHERITANCE OF MITOCHONDRIA IN BIVALVIA

Introduction

Most eukaryotes have maternal transmission of clonally copied mitochondrial DNA (mtDNA) (Birky 2001). The presence of divergent mtDNA sequences within a single individual, heteroplasmy, occurs in a wide range of taxa and usually results from mutations within an individual, transmission of mutated copies from the mother or paternal leakage (reviewed in Hurst and Hoesktra 1994; Rand 2001). Heteroplasmy is thought to be detrimental leading to selection of selfish deleterious elements (in Hurst and Hoesktra 1994, reviewed in Breton et al. 2007) and to cause some human diseases (reviewed in Schapira 2006). In most organisms, heteroplasmy is a transient state, where mutations in some copies of mtDNA drift out in a few generations due to bottlenecks of mtDNA during oogenesis or negative selection on cells carrying a high mtDNA mutation load (Chinnery et al. 2000; Cree et al. 2008; Khrapo 2008). In some taxa, single-nucleotide polymorphisms in the mitochondrial DNA have become fixed (Doublet et al. 2008; McLeod and White 2010). In contrast, a number of bivalve species have highly divergent mitochondrial heteroplasmies, with a 20-50% difference between

two types of mtDNA found within a single individual (Passamonti et al. 2003; Mizi et al. 2005; Breton et al. 2006; Theologidis et al. 2008; Cao et al. 2009).

Heteroplasmy in bivalves is due to an unusual type of mitochondrial inheritance, where females inherit mitochondria only from their mothers and males inherit mitochondria from both their mother and father, thus possessing two types of mitochondria, the female (F) mitotype and the male (M) mitotype (Skibinski et al. 1994a, 1994b; Zouros et al. 1994a; 1994b; and Zouros 2000). This type of mitochondrial transmission is called Double Uniparental Inheritance (DUI) (Zouros et al. 2000) and appears to be widespread throughout the Class Bivalvia, as it has been found in seven bivalve families (Theologidis et al. 2008). Heteroplasmy has been extensively studied in the Mytilidae (Skibinski et al. 1994a; 1994b; Zouros et al. 1994a; 1994b; and Zouros 2000; Hoeh et al. 1996; 1997), Unionidae (Hoeh et al. 1996; Liu et al. 1996), and Veneridae (Passamonti et al. 2003) where experiments have clearly documented the nature of DUI.

The paternally inherited mitochondria are concentrated in the gonads of males (Garrido-Ramos et al. 1998; Dalziel and Stewart 2002; Cao et al. 2004; Cogswell et al 2006) although they may be sporadically detected in male and female somatic tissues (Passamonti et al. 2003; Garrido-Ramos et al. 1998; Dalziel and Stewart 2002; Obata et al. 2006). The sperm only contains the male type (Skibinski 1994; Venetis et al. 2006). The male type enters the eggs through the sperm but is destroyed in those individuals

destined to become females (Sutherland et al. 1998), though the occasional presence of trace amounts of M mitotypes in females indicates this process may sometimes be incomplete (Zouros 2000).

The origins of DUI within the bivalves is unknown (Theologidis et al. 2008), but probably evolved early despite being found only in distantly related families (Theologidis et al. 2008; Hoeh et al. 1997). DUI is thought to either be lost or has yet to be detected in other bivalve families (Theologidis et al. 2008). Differences exist in the evolutionary pattern of DUI within distant bivalve families. Phylogenetic analysis of the superfamily Unionidea shows male types from different families cluster together separately from the female types from those families, suggesting the origins of the male type precedes the origin of the superfamily (Theologidis et al. 2008; Hoeh et al. 1996; Hoeh et al. 2002; Curole and Kocher 2005). In Mytilids, the male and female types of a species or closely related species generally cluster together, but the associations are not as deep as in the Unionidae (Theologidis et al. 2008; Hoeh et al. 1996; Hoeh et al. 2002). The different patterns in Mytilids may result from the presence of recently "masculinized" M types where a female type becomes entrained into the male transmission line (Zouros 2000; Hoeh et al. 1996; Hoeh et al. 2002). These recently masculinized forms have mostly F related sequences except for the control region, which resembles the control region in males (Cao et al. 2009; Rawson 2005; Burzynski et al. 2006). There is also recombination between the male and female types in Mytilids (Burzynski et al. 2006; Ladoukakis et al. 2001; Rokas et al. 2003). In the Unionidae

there is an absence of recombination or masculinization (Hoeh et al. 2002) perhaps because the male mitotype has a longer COII gene, which prevents recombination (Curole and Kocher 2002; 2005; Chakrabarti et al. 2006). These differences initially led to the hypothesis that heteroplasmy has multiple origins in the Bivalvia (Hoeh et al. 1996). However, because heteroplasmy is such a complex and unique phenomena, and is widespread in the Bivalvia, this is unlikely (Theologidis et al. 2008; Hoeh et al. 1997). Heteroplasmy was discovered relatively recently, so much remains to be learned about the origins, taxonomic distribution, maintenance, and function of DUI (Theologidis et al. 2008; Passamonti and Ghiselli 2009).

So far heteroplasmy has not been found in the most basal bivalves the protobranchs, but very few have been analyzed genetically. Previous phylogeographic studies with formalin fixed specimens of a common abyssal protobranch bivalve *Ledella ultima* (Etter et al. 2005; 2011) occasionally yielded 16S fragments that were readily amplified, but where the sequenced products were problematic, with extensive double peaked reads. Further analyses of this species in fresh specimens utilizing universal primers yielded both previously observed and highly divergent 16S sequences among individuals from the same location. These two results suggested that *Ledella ultima* might be heteroplasmic with male and female mitochondrial haplotypes. Alternative explanations for why divergent 16S sequences might be found in individuals of the same species from the same location include the possibility of cryptic species, the presence of a nuclear copy of mitochondrial DNA (Numt) or contamination. Here we test whether the

divergent haplotypes between individuals of *Ledella ultima* could be found within individuals suggesting heteroplasmy and explore whether heteroplasmy exists in other species within the Ledellinae subfamily and two Nuculanid species.

The Nuculanidae subfamily Ledellinae is a diverse group of tiny bivalves, endemic to the deep sea, and widespread both geographically and bathymetrically (Filatova and Schileyko 1984). Sequencing of the 16S mitochondrial gene from several species yields strong evidence of heteroplasmy in two Ledellinae species. The results provide the first evidence of heteroplasmy in the protobranchs (palaeotaxodonta) and suggest a much earlier origin of DUI than previously thought (Theologidis et al. 2008; Doucet-Beaupré et al. 2010).

Methods

Nuculanidae species were obtained from the following sources: specimens of *Ledella ultima* and *L. sublevis* were collected from the North American Basin (NAB) in June 2008 (research cruise EN147), frozen specimens of *L. pustulosa pustulosa* and *L. p. marshalli* from the West European Basin were provided by John Gage (Fuiman et al. 1999), ETOH preserved *L. ecaudata* from the Antarctic were provided by Katrin Linse and Craig McClain (ANDEEP II), *Nuculana minuta* and *N. pernula* collected from the Skagerat in June 2009 were provided by Rob Jennings. Whole DNA was extracted from individuals using a Qiagen minikit. Some specimens of *Nuculana pernula* were large enough for separate foot and gonad tissue extractions, which should allow for easier

detection of heteroplasmy because male gonads should contain a different mitochondrial type than somatic tissues. Formalin-fixed Ethanol preserved (FFEP) specimens of *L. ultima* from Woods Hole Oceanographic Institute North American Basin stations 77, 70, and 92 and South Hampton Oceanographic Center, West European Basin stations 52216 and North African stations 10148 and 8528 were extracted as described in (Boyle et al. 2004). All frozen or ETOH preserved specimens were PCR screened with a series of primer combinations (Table 3-1)- universal primers, universal forward or reverse with a family specific primer, or two family specific primers. For *Ledella ultima,* the universal primer 16aR (Kocher et al. 1989) and a *Ledella* (or Nuculanidae specific) primer Lu16r4 (Chase et al. 1998a), yielded divergent approximately 400 base pair fragments from the 3' half of the 16S locus. For *L. sublevis,* the primer combination 16aR and Lu16R4 yielded a 400 bp fragment and the primer combination Led16Alt2F with Lu16R4 yielded divergent 300 bp fragments. "Male" and "female" specific primers were developed using an alignment of the divergent sequences from each species.

Forward and reverse primers for the male (Led16MaF/Led16MaR) and female type (Led16FaF/Led16FaR) were used in separate reactions to PCR amplify fragments of the 16S gene for *Ledella ultima*. PCR was conducted in 50 µl reactions consisting of 2 µl undiluted DNA, 10 µl Promega Go Taq flexi buffer, 5 µl 25mM Mg Cl_2, 2.5 µl BSA, 1 µl each primer, 1 µl PCR Nucleotide mix, 0.3 µl Promega Hot Start Taq and H2O (for fresh material) or 10 µl undiluted DNA, 10 µl Promega Go Taq flexi buffer, 5 µl 25mM $MgCl_2$, 2.5 µl BSA, 1 µl each primer, 1 µl PCR Nucleotide mix, and 0.5 µl Promega

Hotstart Taq (for FFEP specimens). All extractions and PCR amplifications of FFEP samples were conducted with separate equipment and reagents in a separate room to prevent spurious results due to contamination. PCR reaction conditions were: initial 2 min denaturation at 94°C followed by 5 cycles of 1 min at 94°C, 1 min at 54°C, 1 min at 72°C, then 35 (or 40 for FFEP) cycles 30s at 94°C, 30s at 55°C, 30 s at 72°C. For *Ledella sublevis*, the "female" Ls16a1F and "male" Ls16b1F primers were used in combination with the reverse primer Lu16R4 in separate reactions. PCR was conducted in 50 µl reactions consisting of 2 µl undiluted DNA, 10 µl Promega Go Taq flexi buffer, 5 µl 25mM Mg Cl_2, 2.5 µl BSA, 1 µl each primer, 1 µl PCR Nucleotide mix, 0.3 µl Promega Hot Start Taq and H2O. PCR reaction conditions were: initial 2 min denaturation at 94°C followed by 35 cycles of 1 min at 94°C, 1 min at 55°C, 1 min at 72°C.

For both *Ledella ultima* and *L. sublevis* the nuclear genes 18S, 28S and H3 were also amplified from a subset of individuals where divergent 16S sequences had been obtained. For 18S the protobranch specific primers Proto18sA2f and Proto18bb2r (Chapter 4) were used with the following PCR reaction conditions: initial 2 min denaturation at 94°C followed by 35 cycles of 1 min at 94°C, 1 min at 54°C, 1 min at 72°C, then one cycle of 72°C for 5 minutes. To amplify the 28S gene the primers 28a (Giribet et al. 2006) and 28SRA (Chase unpublished) were used with the following PCR conditions initial 2 min denaturation at 94°C followed by 5 cycles of 1 min at 94°C, 1 min at 56°C, 1 min 30 sec at 72°C, then 30 cycles of 1 min at 94°C, 1 min at 59°C, 1 min 30s at 72°C, followed by one cycle of 72°C for 10 min. For the H3 gene we used

the primers H3F and H3R (Colgan et al. 2000) with the following PCR conditions: initial 2 min denaturation at 94°C followed by 35 cycles of 1 min at 94°C, 1 min at 54°C, 1 min at 72°C, then one cycle of 72°C for 10 min. Positive PCR results were submitted for sequencing at Agencourt (Beverly, MA) or MGH (Cambridge, MA) to confirm identification. Sequences were submitted to GenBank (Accession numbers HQ907887-HQ907914).

Data Analysis

Sequences were edited and initially aligned in Sequencer ver 4.8. (Gene Codes) Alignments of within species 16S were adjusted by eye within MacClade4 (Maddison and Maddison 2003). For *Ledella ultima* and *L. sublevis,* MEGA 4.1 was used to calculate K-2P distance between "male" and "female" 16S sequences, transition /transversion ratios and to create a Neighbor-Joining tree (Tamura et al. 2007) with *Nuculana minuta* (GenBank accession number DQ280030) as an outgroup. A χ^2 was used to test for differences in base frequencies between "male" and "female" sequences.

To evaluate the relative timing of DUI evolution within *Ledella*, we used a phylogenetic analysis of the "male" and "female" 16S sequences of *Ledella ultima* and *L. sublevis* combined with sequences from additional congeners *L. pustulosa pustulosa*, *L. p. marshalli,* and *L. ecaudata*. All sequences were aligned using MUSCLE (Edgar 2004), and adjusted by eye within MacClade4. Maximum likelihood (in PhyML (Guidon and Gascuel 2003)) and Bayesian phylogenies (in BEAST (Drummond and Rambaut

2007)) were inferred from the 16S sequences using the HKY+G model as determined by jModelTest (Posada 2008) with *Yoldiella inconspicua inconspicua* as an outgroup.

Hypothesis testing

If heteroplasmy exists, divergent haplotypes of mitochondrial genes should be found within a male individual, but not females. Thus, the predicted pattern for heteroplasmy should be that the "male" 16S primers amplify fragments from some individuals but not all and the "female" 16S primers amplify fragments from all individuals. In contrast, if the divergent 16S sequences are from a nuclear mitochondrial pseudogene, both sets of primers should work on all frozen or ETOH preserved individuals, but are unlikely to work on Formalin-Fixed Ethanol Preserved (FFEP) samples. Numts are typically singular copied mitochondrial genes in the nucleus (Bensasson et al. 2001). It is difficult to reliably amplify single copy nuclear DNA from FFEP samples, especially for minute protobranch clams collected >40 years ago (Boyle et al. 2004). Additional indicators of Numts might be disruption of the secondary structure of the 16S rRNA or a reduced transition/transversion bias (Bensasson et al 2001; Pons and Vogler 2005; Olson and Yoder 2002).

Results

Ledella ultima

For *Ledella ultima,* both "male" (289 bp) and "female" primers (319 bp) successfully amplified mtDNA. In all individuals where the "male" primers amplified, the "female" primers also amplified, but for others only the female primers worked

(Table 3-2). The ratio of individuals with both types to individuals having just one type is 7/5, which is reasonably close to a 1/1 ratio. For both sets of primers, PCR yielded positive results for frozen, ETOH preserved and FFEP samples (some of which were collected >40 years ago). Nuclear genes (18S, 28S, and H3) were not divergent between a subsample of individuals with divergent 16S sequences.

Although the sex of individuals cannot be confirmed, for clarity heteroplasmic individuals are referred to as males and homoplasmic individuals as female because the reverse is unlikely (Zouros 2000). Male and female sequences of 16S from *Ledella ultima* were quite divergent (K2P distance of 0.301 Table 3-2) and cluster separately on a neighbor joining tree with female haplotypes more distant (K2P distance of 0.334 Table 3-2) from the outgroup *Nuculana minuta* (Figure 3-1). There is no significant change in base pair composition between the 16S fragment of males and females in *L. ultima* (Table 4; M vs F χ^2= 0.585 df =3). In all cases, as is typical of molluscan mtDNA, the sequences were AT rich. Transition /transversion bias for *L. ultima* is R= 3.108.

Ledella sublevis

The primer combination LedAlt2F and Lu16R4 yielded divergent sequences for *Ledella sublevis*. Males were considered those individuals where both the Ls16b1F primer and the Ls16a1F primer worked, while those in which only the Ls16a1F primer amplified were considered “females”. Male and female 16S sequences (166bp) from *L.*

sublevis were less divergent than those in *L. ultima* (K2P distance of 0.145, see Table 3-3). As with *L. ultima*, the male type of *L. sublevis* was more similar to the outgroup *Nuculana minuta* (K2P distance of 0.459, see Table 3-3). Male and female types form separate clades in a neighbor-joining tree (Figure 3-2). The ratio of individuals with both types (males) to those with one type (females) is 1/1 (Table 3-2). There is no significant change in base pair composition between the 16S fragment of males and females in *L. sublevis* (*L.s.* M vs F χ^2= .0972 df=3 p>0.05). Transition /transversion bias for *L. sublevis* is R= 16.354 which is considerably greater than *L. ultima*. The nuclear genes (18S, 28S, and H3) were identical between individuals with divergent 16S sequences.

Other Ledella species

Only one type of 16S sequence was found in *Nuculana minuta* and *Nuculana pernula* even for DNA separately extracted from the gonads. Of course, this does not rule out the presence of heteroplasmy in *Nuculana spp.* because the second type may be too divergent to amplify with the same primers (Theologidis et al. 2008)

Phylogenetic Analysis:

The *Ledella ultima* male and female haplotypes form a strongly supported monophyletic group separate from all the other Ledella species (Figure 3-3). Male and female haplotypes of *Ledella sublevis* also form a well-supported monophyletic group, separate from *L. ultima* and contained within a clade of other congeners (Figure 3-3).

Discussion

The presence of two 16S haplotypes within individuals of *Ledella ultima* and *Ledella sublevis* most likely represents male/female mitochondrial heteroplasmy, as found in seven other bivalve families (Theologidis et al. 2008). This is the first record of heteroplasmy in the protobranchs, the most basal lineage within the bivalves (Giribet and Wheeler 2002).

Evidence for Heteroplasmy

The primary evidence for establishing heteroplasmy in early studies was the difference in mtDNA sequences between males and females e.g. (Liu et al. 1996; Fisher and Skibinski 1990; Hoeh et al. 1991). Subsequent experiments where males and females with known mitotypes were crossed confirmed the presence and nature of DUI, especially in Mytilids e.g. (Cree et al. 2008; Skibinski et al. 1994a; Zouros et al. 1994a; Garrido-Ramos et al. 1998; Cogswell et al. 2006; Kechington et al. 2002; 2009). Unfortunately, because deep-sea protobranchs are difficult to keep alive and probably have very long generation times (Turekian et al. 1975), experimental confirmation of DUI is impractical.

Although we cannot experimentally confirm the presence of DUI, the evidence presented here strongly suggests heteroplasmy occurs in two species of the genus *Ledella*. The recovery of two separate and unique16S sequences from a single individual combined with the lack of variation at nuclear loci (18S, 28S, and H3) is consistent with heteroplasmy and rules out alternative explanations involving

contamination or cryptic species. Contamination is unlikely because the "male" sequences are unique, meaning they are not similar to any of our other amplified sequences or to those deposited in GenBank, they were retrieved independently from samples processed with separate equipment and reagents in a different room, and our negative controls were blank. The consistent amplification of two 16S sequences from a single individual, and the lack of variation at nuclear loci suggest the divergent sequences do not represent cryptic species.

Several lines of evidence suggest the divergent sequences are not Nuclear mitochondrial pseudogenes. The presence of divergent 16S genes within individuals from multiple species within the same family, but not from all individuals of these species is more likely to represent heteroplasmy. If one of the two 16S sequences was a Numt, we would expect the sequence to amplify in most individuals, but be less likely to amplify in FFEP specimens. Single copy nuclear genes generally do not amplify from FFEP tissues, because formalin degrades DNA so the chance you can amplify DNA decreases significantly for single copy genes. We found neither of these expectations to be true. The incidence of reported pseudogenes from Mollusca is low (Bensasson et al. 2001). A recent BLAST search using the terms "Mollusca and pseudogene" yielded relatively few hits (30) compared to other taxa (e.g. crustaceans (Buhay 2009)), and most of these matched COI or tRNAs. Phylogenetic analyses of pseudogenes are likely to produce interspecific monophyletic clades of male (or female) haplotypes, but the male and female 16S sequences from *L. ultima* and *L. sublevis* form intraspecific clades

(Figure 3-3). Also, if the "male" sequence was a Numt, we might expect to find homology between the "male" sequences from *L. ultima* and *L. sublevis,* such as long deletions (Pons and Vogler 2005), though none were detected. Finally, the ratios of heteroplasmic/homoplasmic individuals found in both species are similar to known sex ratios of protobranchs, which generally are even (Zardus 2002).

Divergence of Male and Female Mitotypes

The degree of divergence between the male and female 16S haplotypes (40% in *Ledella ultima* and 15% in *L. sublevis*) is within the range of that found for bivalves with DUI, but far exceeds what we typically find among conspecifics for deep-sea protobranchs throughout the Atlantic (Etter et al. 2005; 2011; Chase et al. 1998b; Zardus et al. 2006). Geographic and bathymetric divergence of 16S haplotypes among conspecifics vary with depth, but are generally less than 10% Atlantic (Etter et al. 2005; 2011; Chase et al. 1998b; Zardus et al. 2006). Levels of divergence in whole genome analyses of the two gender-associated mitotypes in other species was similar to that for the protobranchs (e.g. 50% in *Inversidae japanensis* Unionidae; 34% in *Venerupis philippinarum* Veneridae; 37% in *Donax trunculus* Donacidae (Theologidis et al. 2008). The average in Mytilids was about 20% with the difference in 16S being 16% (Mizi et al. 2005; Breton et al. 2006, Cao et al. 2009) while in the Venerid, *Tapes philippinarum,* the divergence was 15% (Passamonti et al. 2003).

The high rate of divergence between male and female mitotypes in bivalves suggests separation over evolutionary time scales and possibly relaxed selective constraints, particularly in males, where its function is primarily restricted to gonad tissues (Passamonti et al. 2003; Breton et al. 2006; Zouros 2000; Stewart et al. 1996). A variety of mechanisms have been suggested for faster evolution of male mitotypes including relaxed selection, higher mutation rates, the smaller population size of the M mtDNA and the higher rate of M mtDNA duplication during spermatogenesis (Zouros 2000; Passamonti and Ghiselli 2009; Stewart et al. 2006). A faster rate of evolution in the male mtDNA would enhance the rate of divergence from female mtDNA (Passamonti et al. 2003; Liu et al. 1996; Stewart et al. 1995; 1996; Rawson and Hillbish 1995; Quesada et al. 1998).

Phylogenetic Implications

Mitochondrial heteroplasmy is widely distributed within the Bivalvia, occurring in seven families and five superfamilies (Theologidis et al. 2008). Its presence in the protobranchs indicates DUI is more widespread and originated much earlier than previously thought. Although widespread, it has not been detected in many lineages closely related to those where it has been found. For example, although it has been documented in *Venerupis philippinarium* (Passamonti and Scali 2001), it has not been detected in other congeners (Theologidis et al. 2008), or well-studied genera within the Veneridae (e.g. *Gemma*, *Mercinaria*, *Venus*). Two explanations have been advanced to account for the broad but sporadic phylogenetic occurrence of DUI and its absence from

many intermediate lineages. The first, referred to as the multiple origins hypothesis, suggest DUI evolved independently in each of the lineages where it has been documented. Many consider the multiple origins hypothesis to be unlikely because the number of independent origins increases with the number of separate lineages where heteroplasmy has been identified (Hoeh et al. 2002; Walker et al. 2006; Theologidis et al. 2008; Doucet-Beaupré et al. 2010).

An alternative explanation, the early origin hypothesis, suggests DUI evolved early in the evolution of the bivalves and has subsequently been lost from many taxa, or simply has not been detected. Most consider this to be the more likely explanation, although the phylogenetic relationships among the male and female mtDNA appear inconsistent with this hypothesis. If DUI evolved once early in the origin of the bivalves, phylogenetic analyses of those taxa with DUI should produce a single intertaxon clade of male mtDNA and a separate clade of all female mtDNA. Recent phylogenetic analyses of mtDNA from taxa exhibiting DUI did not find bivalve-wide gender specific clades (Theologidis et al. 2008; Doucet-Beaupré et al. 2010). Instead, phylogenetic patterns were complicated and indicated multiple levels of association between male and female mtDNA within and between different taxa. Across multiple families within the Unionoidae male and female mtDNA formed gender-specific monophyletic clades (Breton et al. 2007; Hoeh et al. 2002; Doucet-Beaupré et al. 2010) and this was also true for some mytilids (Breton et al. 2007; Theologidis et al. 2008). Across other Autolamellibranchia (Breton et al. 2007; Theologidis et al. 2008) and

within the protobranchs (Fig. 3-3) male and female mtDNA form intraspecific clades separate from other taxa. The formation of intraspecific clades or intrataxon (genus, family or superfamily) clades of male and female mtDNA appears inconsistent with a single origin, and more indicative of multiple independent origins. However, masculinization events, where female mtDNA become substituted for male mtDNA in males, could easily mask the origin of DUI and create the complicated pattern of gender-specific phylogenetic relationships observed (Hoeh et al. 1997; 2002; Theologidis et al. 2008; Doucet-Beaupré et al. 2010). At this point it is difficult to use the phylogenetic relationships among the male and female mtDNA to definitively arbitrate between multiple or early origin of DUI, but the presence of DUI in the basal branch of the bivalves suggests an ancient origin.

The sporadic occurrence of DUI throughout the bivalves is difficult to interpret. At least three possible interpretations exist for the absence of DUI in any particular lineage 1) it never evolved, 2) it was lost, or 3) it has not been detected. As others have argued, detection of DUI is a significant problem because male mtDNA appear to evolve rapidly potentially precluding amplification with universal primers (Hoeh et al. 1997; 2002; Theologidis et al. 2008; Doucet-Beaupré et al. 2010) or even taxon specific primers targeting female mtDNA. It is also unclear how many taxa have been studied in sufficient detail to be sure DUI is absent. For the protobranchs we considered, DUI was detected in two species but undetected in other species. It is not clear whether the inability to detect heteroplasmy in these five species indicates that DUI is absent, or that

the PCR-based assay we used was not flexible enough to detect the male mitotypes. As with other DUI studies, interpreting the absence of DUI remains problematic.

Conclusions

Mitochondrial heteroplasmy appears to be present in protobranch bivalves supporting the hypothesis that DUI evolved early in Bivalvia with subsequent loss from some families. The presence of heteroplasmy in the Nuculanids significantly predates prior estimates of the origin of DUI (Theologidis et al. 2008; Doucet-Beaupré et al. 2010). Previous work documented DUI in both the Paleoheterodonta and the Pteriomorpha suggesting that it might have evolved in the branch leading to the Autolamellibranchia, about 460 Mya (Little and Vrijenhoek 2003). The occurrence of heteroplasmy in the protobranchs (palaeotaxodonta) suggests a much earlier evolution, perhaps in the early Cambrian when the bivalves are thought to have evolved from rostroconch molluscan ancestors (Waller 1998).

The nature of how DUI evolved and why it persists remains unknown (Passamonti and Ghiselli 2009). There is some suggestion that the different mitochondrial types play a role in sex determination (Saavedra et al. 1997; Zouros 2000; Passamonti and Ghiselli 2009; but see Kenchington et al. 2002;2009), and if true protobranchs may be important for uncovering the nature of sex determination in bivalves. Another hypothesis for the evolution of DUI is that the M-type mitochondria affect sperm function. However, recent experiments in Mytilids have shown the M-type sperm are

slower than recently masculinized M-types (Jha et al. 2008). Whatever its function, the detection of heteroplasmy in protobranchs provides an opportunity to study the early evolution of DUI.

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TABLE 3-1: List of primers used in this study

Primer name	Sequence 5' to 3'	Reference
General primers for 16S Nuculanidae		
16Sar	ATGTTTTTGATAAACAGGCG	Kocher et al. 1989
Lu16R4	GCTGTTATCCCTCCAGTAAC	Chase et al. 1998a
Led16Alt2F	CCGTYCAAAGGTAGTGTAAT	This study
LANA316RB	AGCTCAGTTGCCCCAACTAAA	This study
Ledella ultima M/F specific for 16S- Forward primers also work with Lu16r4		
LedFa16f	AGTTCCTGCTCAATGATAATAA	This study
LedFa16R	CCAGTTGCCCCAACTAAAATT	This study
LedMa16F	TTCTGCTCAATGGTGTRCG	This study
LedMa16R	AAACACACCATAAGCCAAAAC	This study
Ledella sublevis M(b)/F(a) specific for 16S work with Lu16R4		
Lsa116F	TTTTATGAAAGAAGAATTTAACTTTGC	This study
Lsb116f	GTTTATGAAAGTAAAAATTAACCTTGT	This study
18S primers –protobranch specific		
Proto18sA2f	ATGCATGTCTAAGTACANACT	This study
Proto18bb2r	AACCACGGTAGGCATATCA	This study
28S primers		
28sa	GACCCGTCTTGAAACACGGA	Giribet et al. 2006
28SRA	GAA AAG ARA ACT CTT CCC GG	Chase *unpublished*
H3 primers		
H3F	ATGGCTCGTACCAAGCAGACVGC	Colgan et al. 2000
H3R	ATATCCTTRGGCATRATRGTGAC	Colgan et al. 2000

TABLE 3-2: Summary of results from PCR of individuals of *Ledella ultima* and *L. sublevis* with male and female specific primers.

Species	#ind. w/ male and female haplotypes	#ind. w/ only female haplotypes	#stations	# basins
Ledella ultima	7	5	5	3
Ledella sublevis	3	3	3	2

TABLE 3-3: Net K2P distances between male and female type for *Ledella ultima* and *Ledella sublevis* compared with an outgroup *Nuculana minuta*.

Species	Distance M vs F	M vs outgroup	F vs outgroup
Ledella ultima	0.301	0.256	0.334
Ledella sublevis	0.166	0.459	0.548

TABLE 3-4: Average percentage of each base in 16S fragments from each species. For *Ledella ultima* χ^2=0.585, df=3 p>0.05. For *Ledella sublevis* χ^2=0.097, df=3, p>0.05

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Species/gender	%T	%C	%A	%G
L. ultima F	36.6	9.8	34.9	18.7
L. ultima M	35.6	10	31.6	22.8
L. sublevis F	36.8	9.5	34.4	19.3
L. sublevis M	35.2	9.0	36.2	19.6

FIGURE 3-1: Neighbor-joining tree based on K-2P distances showing the separate clades of Male and Female haplotypes of *Ledella ultima* in relation to the outgroup *Nuculana minuta*.

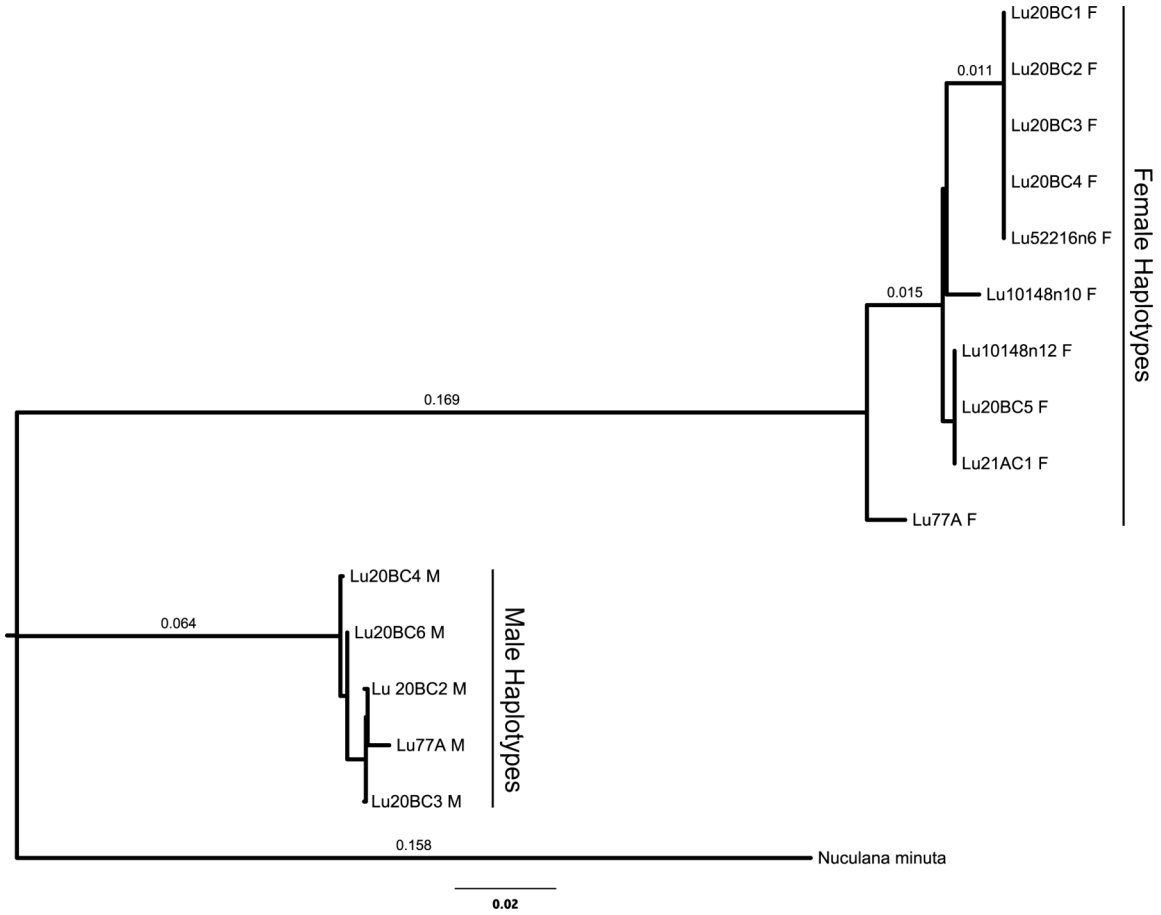


FIGURE 3-2: Neighbor-Joining tree based on K-2P distances showing the relationship between male and female 16S types of *Ledella sublevis* in relation to the outgroup *Nuculana minuta*.

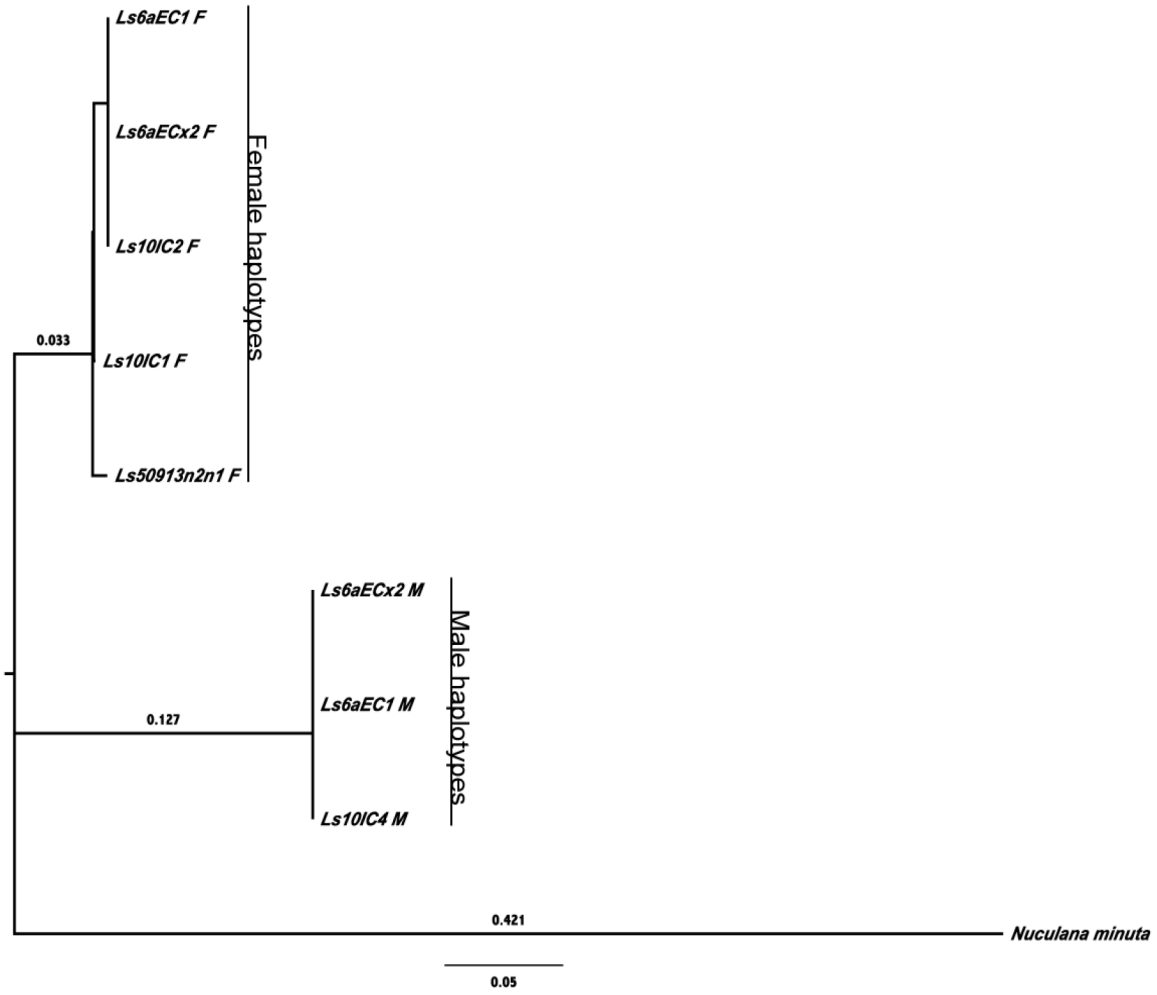
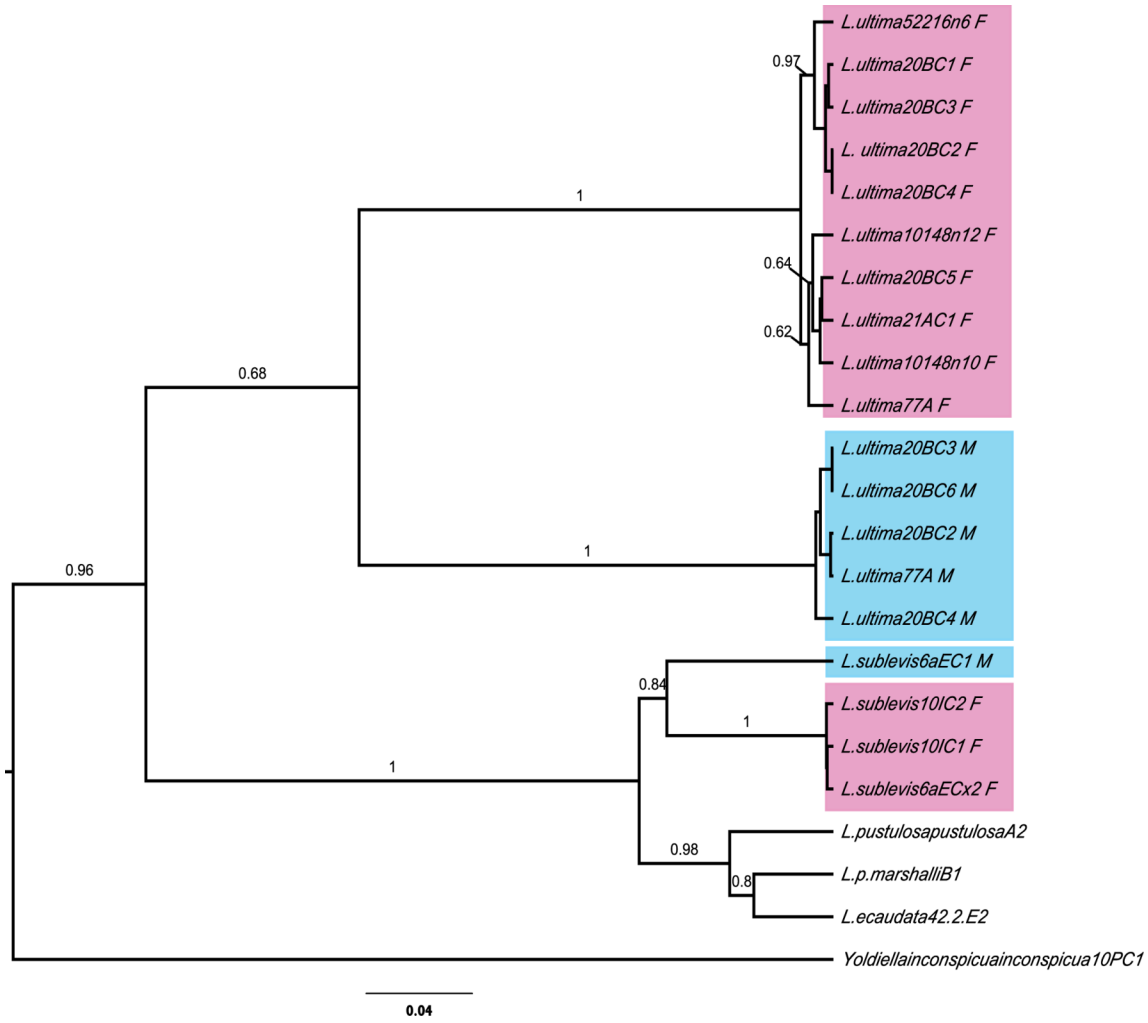


FIGURE 3-3: Bayesian tree of *Ledella spp.* based on 16S sequences. “Male” clades are highlighted in blue, “female” in pink. Branch labels indicate Bayesian posterior values >.50.



CHAPTER 4
PHYLOGENY OF THE DEEP-SEA BIVALVE FAMILY LEDELLINAE

Introduction

High species richness in the deep sea is puzzling from both an ecological and evolutionary perspective. How can so many species coexist on limited resources? What factors led to high species richness for many different taxa? Is high species diversity the result of in-situ radiation, accumulation of taxa over long time periods, and/or low extinction rates? The evolution the deep-sea fauna is poorly understood, in part, because few have been analyzed phylogenetically. Some deep-sea taxa appear to be relics (Menzie et al. 1973; Kiel and Little 2006), but evidence also suggests some taxa are derived from recent immigrations and in-situ radiations. There are also many cryptic species (Chase et al. 1998a; Etter et al. 1999; Raupach et al. 2007; Vrijenhoek 2008), that require molecular methods to detect. This chapter addresses biogeographic and evolutionary questions about an endemic deep-sea bivalve subfamily, the Ledellinae using DNA from a combination of formalin-fixed museum specimens and fresh material.

How have historical events affected the evolution of the deep-water fauna? Ocean-wide deep anoxic conditions during the Cenomanian/Turonian boundary (aprx. 90 mya) and Paleocene (aprx. 65 mya) stages may have caused the extinction of much of the

deep-sea benthos (Wolff 1960; Menzies et al. 1973; Jacobs and Lindberg, 1998). Consistent with this notion, some deep-sea fauna such as ostracods and Flabellifera isopods appear to be recently derived from shallow water ancestors (Wilson 1999). However, records of bioturbated sediments from high latitudes, that date back at least 90 million years, indicate parts of the deep sea were oxygenated (Horne 1999). Asellote isopods have radiated extensively in the deep sea and have greatly diverged from shallow-water isopods, which implies they have a long history in the deep sea and survived the anoxic periods (Hessler and Thistle 1975; Wilson 1999; Raupach et al. 2009). Deep-water corals are ancestral to several groups of shallow corals; these emergence events occurred at least 65 mya, suggesting the deep-sea species are much older (Lindner et al. 2008; Kitahara et al. 2010). Some echinoid species show evidence of a long history in the deep, while others have more recently migrated during periods of climatic change (Smith and Stockley 2005). Clearly all organisms were not equally affected by these anoxic events. Periods of ocean-wide anoxia may have isolated populations within deep oxygenated refugia, facilitating speciation (White 1987; Rodgers 2000).

The evolutionary and biogeographic history of protobranch bivalves has not been studied. They are the most basal extant group within the Bivalvia (Giribet and Wheeler 2002; Giribet et al. 2006) and are more diverse and abundant in the deep sea than in shallow water, often comprising 70% of the bivalves in a sample (Allen 1978; 1979). Unlike most bivalves, they are deposit feeders with highly modified hind guts, which probably make them better adapted for existing on the meager food supplies at great

depths (Allen 1978; 1979). They grow slowly, reproduce continuously and most are thought to have lecithotrophic larval dispersal (reviewed in Zardus 2002; Zardus and Martel 2001). Although little is known about the phylogenetic affinities of protobranchs, Maxwell (1988) has proposed a preliminary evolutionary relationship between the families and subfamilies (Figure 4-1).

The subfamily Ledellinae was used to test hypotheses about colonization and speciation patterns within the Atlantic because it is endemic to the deep sea, relatively species rich, and found throughout the world's oceans. This group is well known taxonomically with detailed morphologically based taxonomies for both the Atlantic (Allen and Hannah 1989) and Pacific (Filatova and Schileyko 1984) forms. The Ledellinae are one of the more species rich protobranch groups in the Atlantic. The genera *Ledella* and *Spinula* are considered by some to be in separate subfamilies (Allen and Sanders 1982), while others classify them into a single subfamily (Filatova and Shylecko 1984). According to Allen and Sanders (1982) the Nuculaninae, is the sister taxon to the Ledellinae and Spinulinae and, thus, should be the most appropriate outgroup. However, this hypothesis has never been tested phylogenetically.

To develop molecular phylogenies of deep-water protobranchs it is necessary to rely on a combination of formalin fixed-ethanol preserved (FFEP) and other museum specimens as well as a few ETOH or frozen species. Thus the DNA sequences for the phylogeny are limited to highly copied mtDNA and nuclear rDNA. The fresh samples allowed amplification of DNA using universal or mollusk specific primers and the resulting sequences were used to design family specific primers for the FFEP samples.

Mitochondrial heteroplasmy exists in at least two Ledellinae species (Chapter 3). Clearly the presence of two types of mitochondria within some individuals complicates phylogenetic analysis based on mitochondrial genes. In other bivalve studies (Unionidae), the presence of DUI has been used advantageously as another character to inform the phylogeny (Hoeh *et al.* 2002).

A geographically referenced phylogeny of the Ledellinae can be used to address a number of biogeographic and evolutionary questions. How was the Atlantic colonized? If the Atlantic Ledellinae are monophyletic with respect to Ledellinae from other oceans, it suggests they were derived from a common ancestor that colonized the Atlantic. If the Atlantic Ledellinae are not monophyletic it may indicate multiple colonization events, perhaps by multiple pathways. The *Ledella* species within the Atlantic, with the exception of *Ledella ultima*, are endemic and only occur in a few basins suggesting they radiated within the Atlantic (Allen and Sanders 1997). Did the Atlantic Ledellinae originate from Antarctic, Artic, Pacific, or Tethys species? Allen and Sanders (1997) compiled a comprehensive zoogeographic monograph of the Atlantic protobranchs (from examination of >80,000 specimens) and formulated several hypotheses about their evolution. They argued that the modern distribution of Atlantic protobranchs was influenced by deep currents originating from the Antarctic, and the colonization of the Atlantic by protobranchs had four possible sources: shallow Antarctic, Arctic, Pacific and the Ancient Tethys Sea. Based on the distribution of *Ledella ultima*, *Ledella pustulosa*, and *Ledella aberrata* they suggest these species originated from the Tethys Sea or the

South Atlantic. The phylogenetic relationships among the Ledellinae should provide a test of the colonization of the Atlantic.

Methods

Materials

Specimens from the subfamily Ledellinae: including the genera *Ledella*, *Tindariopsis*, and *Spinula* were acquired from a variety of locations and sources. Most of the Atlantic species, the only known Antarctic *Ledella* species, a few species from the Pacific were acquired for the *Ledella* genus. Four species from the Atlantic, one species Antarctic and two Pacific species were included for the genus *Spinula* (Table 4-1). Two species from the genus *Tindariopsis* were also included. The majority of specimens were provided by Howard Sanders and John Allen and were collected as part of the Woods Hole Oceanographic Institute sampling program from the 1960s (Sanders 1977). Other samples were obtained during visits to the US Natural History Museum, British Museum of Natural History (BMNH), the South Hampton Oceanographic Center and loans from the Los Angelos County Musuem, and SCRIPPS Institute of Oceanography. The type specimens of many species and the Challenger collection from which many of the deep protobranch species were originally described were examined. Frozen and ETOH preserved samples were acquired from: ANDEEP cruises, BMNH (BP collections), EN447 cruise and a Skagarat cruise.

Characters

Using frozen or ETOH preserved specimens, portions of the 16S, 18S, 28S, and H3 genes were sequenced from species from both the Spinulinae and Ledellinae as well as from a number of outgroups (Tables 4-2 and 4-3). From the FFEP specimens, smaller portions of 16S, 18S and from a limited number of species 28S, were sequenced (Table 4-3). Mitochondrial genes are generally more variable and evolve faster than nuclear genes (Li, 1997; Palumbi, 1996). The use of multiple genes with different rates of evolution improves phylogenetic inferences because variable genes help to resolve relationships between closely related taxa, whereas slowly evolving genes help resolve more distantly related taxa (Klompen et al, 2000). These genes were chosen because they were useful for molluscan phylogenies at a similar taxonomic level (e.g. Koufopanou, et al. 1999; Anderson 2000; Medina and Walsh 2000; Goffredi et al. 2003; Williams et al. 2003).

Extraction

Whole individuals in ETOH or frozen were washed in TBE. DNA was extracted with a Qiagen QIAamp DNA Mini Kit following manufacturers protocol. FFEP and dried samples were extracted with a modified protocol where whole specimens were soaked for 7 days in TBE at 37ºC, extracted using a Qiagen QIAamp DNA Micro Kit® with the following modification to the kit protocol: 8hr in buffer ATL and PK at 56ºC, followed by1 hr 90ºC as recommended for Formalin-fixed Parafilm Embedded samples, then follow the standard protocol.

Primer development

To facilitate amplification of DNA from FFEP specimens primers were developed to target Nuculanidae species from alignments of existing sequences from GenBank. Amplification of DNA from fresh samples used universal primers or combinations of universal and family specific primers. To facilitate the development of specific primers DNA was amplified from ethanol preserved and frozen specimens of two *Ledella* species, one *Spinula* species and other ethanol preserved protobranchs including: *Yoldiella valleti*, *Propeleda longicaudata*, and *Silicula rouchi*. For the Formalin-fixed ethanol preserved and dried samples, taxa specific primers were developed to target gene fragments of approximately 250-400 base pairs per gene.

Previous work in the lab sequenced portions of the 28S and 16S genes from formalin-fixed alcohol-preserved *Ledella ultima* (Chase et al. 1996a). The existing16S primers targeted a hypervariable region in the 3' end of the gene and were intended for population level analysis. For this study a primer was designed to a more conserved region upstream of the hypervariable area (Ledalt2F) and a conserved area within the hypervariable region (lana316Rb). The forward primer was also combined with the previous reverse primer (Lu16R4) for a longer fragment. Primers were also developed for amplification of 18S and were designed to specifically avoid commonly encountered contaminants including human and parasitic hydroids. These primers are intended to work on protobranchs and were designed from an alignment of sequences generated from fresh specimens using universal primers and protobranchs available on GenBank.

Amplification

Using the primers designed for Nuculanidae or protobranchs, PCR reactions were carried out in 50 µl reactions using 10 µl undiluted DNA for FFEP or 2 µl for fresh or frozen samples and 10 µl Promega Go Taq flexi buffer, 5 µl 25 mM Mg Cl_2, 2.5 µl BSA, 1 µl (10 pm) each primer, 1 µl PCR Nucleotide mix, 0.3 µl Promega Hot Start Taq and H2O. For 18S the protobranch specific primers Proto18sA2f and Proto18bb2r were used with the following PCR reaction conditions: initial 2 min denaturation at 94°C followed by 35 cycles of 1 min at 94°C, 1 min at 54°C, 1 min at 72°C, then one cycle of 72°C for 5 minutes. To amplify the 28S gene the primers 28FA and 28SRA; 28a and 28RA; or 28mm and 28ee were used with the following PCR conditions initial 2 min denaturation at 94°C followed by 5 cycles of 1 min at 94°C, 1 min at 56°C, 1 min 30 sec at 72°C, then 30 cycles of 1 min at 94°C, 1 min at 59°C, 1 min 30s at 72°C, followed by one cycle of 72°C for 10 min. For the H3 gene the primers H3F and H3R under the following PCR conditions: initial 2 min denaturation at 94°C followed by 35 cycles of 1 min at 94°C, 1 min at 54°C, 1 min at 72°C, then one cycle of 72°C for 10 min. 5 µl of the PCR reaction were run out on a 1% agarose gel with EtBr to determine if amplifications were successful. Products of PCR amplification were purified using Promega Wizard kit and sent to a sequencing facility (either Agencourt, Beverly, MA or MGH sequencing core, Cambridge, MA).

Analysis

For each gene, sequences were edited and aligned using Sequencher 4.1 and MUSCLE 3.7 (Edgar 2004), or Clustal X ver 2 (Larkin et al. 2007) then adjusted by hand using MacClade 4 (Maddison and Maddison 2000).

Because the phylogeny of the protobranchs is unknown, the best outgroup for the Ledellinae is not certain. A phylogeny of concatenated sequences from the nuclear genes 18S, 28S and H3 was inferred with species representing genera in the family Nuculanidae (*Nuculana, Propeleda, Spinula, Tindariopsis, Ledella*), and using species from the Family Sareptidae (*Yoldia, Yoldiella*), Malletiidae (*Malletia*), Neilonellidae (*Neilonella*), and Siliculidae (*Silicula*) to determine a reasonable outgroup for the Ledellinae. For 18S *Nuculoma granulosa* was used as an outgroup, because the gene is highly conserved

Phylogenies were constructed with PhyML (Guindon *et al*. 2004) using Maximum Likelihood and Bayesian methods using BEAST v1.5.3 (Drummond *et al.* 2009). The best-fit model for nucleotide substitution for each alignment was inferred in jmodeltest (Posada 2008).

Summary of trees:

1. 18S tree using 279 bp with HKY+G, -lnL 786.1046, AIC 1682.3892 (highest choice available in BEAST).
2. 28S tree (Short w/FFEP sequences 205 bp TPM3+I+G –lnL 594.9963; 1273.9925; long with just fresh samples 601 bp)
3. H3 tree 305 bp GTR+1+G jmodeltest –lnL 1009.7607, AIC 2091.5214 model selected.

4. 18S/28S/H3 tree concatenated H3(305 bp)/18S (318 bp)/28S(601 bp); GTR +I+G –lnL 3165.9861; AIC 6391.9721
5. 16S trees: Because the 16S sequences were amplified by different primers and thus were different lengths (Table 4-3) or could be from the male form, 16S phylogenies were inferred with different alignments ranging from using all data, removing the known and suspected male forms of 16S, deleting the short sequences, deleting the short sequences and adding the males. This strategy was used to explore if the topology was robust to including more taxa and data. Some argue to include taxa even when data are missing, because simulations suggest they are often placed correctly and they can break up long branches (reviewed in Wiens 2006). While all sequences could be used, by trimming the ends, very short sequence would result (Table 4-3). Remaining sequences were realigned after removing specified sequences.
    a. all sequences including male types and short sequences (with incomplete ends treated as missing data) model GTR+G -lnL 4111.7077 AIC 8473.4154
    b. alignment with males & suspected males jmodel test HKY+G -lnL 3186.1215 AIC 6562.2430
    c. alignment with male and short sequences removed HKY+G-lnL 2884.3234 AIC 5942.6467
    d. alignment with male and suspected males added and short sequences removed model: GTR +I+G –lnL 2865.3774 AIC 5962.7547

6. 18s/16s concatenated model:HKY+I+G -lnL 2499.2321 AIC 5078.4643

Geographic position of the species was mapped onto the resulting 16S phylogenetic tree with highest support value to test biogeographic hypotheses. Are the Ledellinae in the Atlantic monophyletic suggesting a single colonizing ancestor? If the Atlantic Ledellinae do not form a single clade within the phylogeny, this would suggest multiple colonizers to the Atlantic either from multiple migrations by the same pathway or migration from different pathways. To test for the most likely ancestral pathway, species from the Antarctic, and Pacific were included in the phylogeny. If the resulting phylogenies provide no support for these pathways, one alternative is the Ledellinae may have originated from the ancient Tethys Sea. This alternative may be harder to falsify, but some predictions can be made: e.g., if the Atlantic Ledellinae were derived from an ancient Tethys ancestor they might be more ancestral to Ledellinae species from other oceans.

Using a molecular clock rate under BEAST strict clock model, the hypothesis of timing of the evolution of the Ledellinae was inferred with mutation rate of 0.0016 myr^{-1}, based on the closest known 16S rate for bivalves (Page and Linse 2002). This is a very approximate molecular clock as fossils for deep-sea mollusks are rare, but this value is also similar to that used for other mollusks (Reid et al. 1996). There are many problems associated with using molecular clock to estimate these events so temporal estimates should be interpreted cautiously.

Results

A list of species from which DNA was extracted, amplified and sequenced/gene is presented in Table 4-3. Amplifications were unsuccessful from the following Atlantic species (Boyle et al 2004): *Ledella lusitanensis, L. sandersi* (Allen), *L. solidula*, *L. similis,* or *L. verdiensis*. A list of other protobranchs sequenced was compiled for use as outgroups and for a phylogeny of the Protobranchs (Table 4-4).

The 18S Bayesian tree (Figure 4-2) based on 279bp of the 5' region of 18S shows high support for Ledellinae, with the exception of *Ledella galathea*. *Spinula filatova* and *Bathyspinula calcar* form a clade in the 18S tree but are in different clades in the 16S tree (Figure 4-6), suggesting 16S sequence from *Spinula filatova* have been derived from the male type mitochondria. The Ledellinae are not well resolved, which is consistent with the conservative nature of 18S. Also *Tindariopsis agatheda* as described by Allen (1996) and *Tindariopsis sulcata* as designated by Dall 1889 (or Malletia) are in separate clades with *T. agatheda* grouping with the Ledellinae.

A tree based on 305bp of the nuclear H3 gene using fewer species (Figure 4-3), also shows high support for monophyly of the Ledellinae as defined by Filatova and Shylecko (1984). *Ledella ultima* groups with the Spinula species and forms a well supported clade with *Ledella sublevis* and *Ledella ecaudata* (Figure 4-2). A tree based on a small portion of 28S does not support monophyly of the Ledellinae, but support is low for many of the nodes (Figure 4-3). Using fewer species with a longer portion of 28S did not improve this result (not shown). A concatenated phylogeny of H3/18S/28S provides high support for the Ledellinae (Figure 4-4).

16S: The topologies of the trees resulting from different 16S alignments were more variable but mostly consistent (Figures 4-6 to 4-9). The position of *Ledella ultima*, 2 *Spinula* spp. and *Bathyspinula calcar* varied in terms of which group was basal. The support value increases for the Ledellinae clade when the suspected and known males are removed from the tree and the short sequences were also removed. Removing the short sequences had a greater impact on increasing posterior probabilities especially for the main Ledellinae clade , increasing from 0.57 when all sequences are included (Figure 4-6) to 0.99 when short sequences are removed (Figure 4-9). This is probably due to a better alignment of the remaining sequences providing more stable inferences about evolutionary affinity. 16S is difficult to align because there are regions that are highly conserved to maintain ribosomal structure as well as hypervariable regions. When aligning sequences from different species many gaps have to be inserted. Removing some of the more distant sequences like the male *Ledella ultima*, reduces the gaps and improves the alignment.

Ledella acuminata and *Ledella acinula* were not in the 16S clade of Ledellinae or Nuculanidae. Both had problematic sequences; *L. acuminata* had a short sequence and *L. acinula* had a double peaked sequence in places and cloning proved problematic for this species. Morphological characters of both are shared with other problematic species. Based on 16S, *Ledella parva* and *oxira* were more closely related to the Nuculana than to the Ledellinae. The depth range of these species (643-1493m; 450-1180m) was shallower than other *Ledella* species except *Ledella acuminata* (475-1427m) (Table 4-1).

Discussion

Phylogenies based on both mitochondrial (16S) and nuclear genes (18S and H3) support monophyly of most of the Ledellinae as defined by Filatova and Shyleyko (including the *genera Ledella, Spinula* and *Tindariopsis* species in Allen 1996). A few species originally defined as *Ledella* are supported by the 16S phylogeny, but reclassification is also suggested by their morphology. Within the well-supported Ledellinae clade, the Atlantic species are not monophyletic suggesting multiple colonizations between oceans. The largest data set was from the mitochondrial 16S gene. The phylogenies from this gene show most of the Ledellinae originate from a rapid radiation within the last 65-50 million years (Figure 4-11). However, *Ledella ultima* is a much older, more basal species, as well as all the *Spinula* species. This is similar to other deep-sea taxa where deep-sea clades are comprised of combinations of older species and more recent radiations (Smith and Stockley 2005). With the Ledellinae there is no evidence of recent radiations derived from shallow-waters suggesting deep-water origins.

Geography

The more recent radiation of Ledellinae is represented by species from the Atlantic, Antarctic, and Pacific. The wide-ranging species, *Ledella sublevis* appears to be basal to the other species in the rapid radiation of the Ledellinae. This clade suggests multiple colonization events between the Atlantic and Pacific and from the West European Basin to the Antarctic (Figure 4-11). Estimates predicted on the molecular clock suggest the major radiation took place within the last 65-50 million years, implying the group

originated in the Tethys Sea with immigration into the Atlantic as it opened (Allen and Sanders 1997).

Also, unlike some taxa (octopods Strugnell et al. 2008) there is no evidence for the Southern Ocean origins in the Ledellinae. Colonization of the deep Atlantic occurred by submergence from the Southern Ocean, because cold isothermal conditions exist from shallow to deep water, the flow of deep water begins in the Antarctic and some deep-sea species occur in shallow Antarctic waters (Menzies et al. 1973; Kussakin 1973). For some taxa phylogenetic patterns are indicative of polar submergence (octopods Strugnell et al. 2008). For the Ledellinae, the basal taxa are not from the Antarctic, and Antarctic species are derived. The putative radiation at 50-65 mya (based on the molecular clock) suggests an earlier radiation than for species considered to have migrated into deep waters from the Antarctic (33 mya) and spread via Antarctic deep-water (15 mya) (Strugnell et al 2008).

When all 16S sequences are included (Figure 4-6) a few species are polyphyletic within the major Ledellinae clade. This could be due to incomplete linage sorting, mitochondrial heteroplasmy or cryptic species. It is interesting that despite finding several species in the same clade as *Ledella pustulosa marshalli,* its often confused and overlapping conspecific *Ledella pustulosa pustulosa* was quite divergent. These species co-occur in the West European Basin, and exhibit subtle morphological differences and are separated by depth (Fuiman et al. 1996). This study provides the first evidence that they are also genetically distinct. Specimens of *Ledella ecaudata* from Antarctica and

Ledella pustulosa argentinae from the Argentine Basin formed a clade with *Ledella pustulosa marshalli.* This suggests *Ledella pustulosa marshalli* may be more widespread than previously thought (Allen and Hannah 1996) or there has been incomplete linage sorting. It is also uncertain at this point if there is DUI in these species, which might explain the polyphyly as well. The grouping of *Ledella kermadecensis* from the Pacific with the male *Ledella sublevis* clade suggests the sequence could be a male type (Chapter 3) and/or the specimen may have been misidentified and it is *Ledella sublevis. Ledella sublevis* has not been identified from the Pacific, but the Pacific forms have not been as thoroughly examined as the Atlantic. More variable genes and more individuals from these species would help resolve the species trees (Maddison and Knowles 2006). The prevalence of DUI in this group also needs to be further explored as with *L. ultima* and *L. sublevis* (Chapter 3).

Bathymetry

The basal species in the Ledellinae are the deepest species. Most of the Spinula species (avg. depth 3678 m) and *Ledella ultima (*avg. depth 4163 m) are abyssal. The species within the more recent radiation of the Ledellinae are bathyal (avg. depth 2978 m) consistent with the hypothesis that speciation rates are greater on continental margins (Etter et al. 2005; Rex and Etter 2010). Given the deep divergence of *Ledella ultima* from extant shallow-water species it is difficult to determine the timing and geographic origins of *Ledellinae* into the deep sea. Others have suggested diversification as early as the Phanerozoic 400 mya (Allen 1979). *Ledella ultima's* divergence from other

Ledellinae species may be due to a slower evolutionary rate in abyssal species (Etter et al. 2011).

Timing

A molecular clock rate based on timing in a shallow water non-protobranch bivalve species was used, obviously independent dating with fossil data would be better as with octopods (Strugnell et al. 2008); however, internal features that are so critical to characterize these species and DNA are not preserved in fossils. Perhaps with more taxonomic sampling at the molecular level, subtle external morphological features could be resolved to better identify fossil species. Considering those caveats, it is suggested by the 16S phylogeny that *Ledella ultima* and the *Spinula* species diverged from the common ancestor long ago, well before the anoxic periods (65 and 90 mya) thought to cause extinction of most of the deep-sea taxa (Jacobs and Lindberg 1998). The rest of the Ledellinae, has had a more recent radiation around 65-50 mya following ocean-wide anoxic periods. This mixed pattern of relic taxa and more recent radiations is not inconsistent with that found for other deep-sea taxa (Stockley and Smith 2005), and may reflect a general pattern in the deep sea. However given the nature of the tools used to date this phylogeny it is difficult to confidently speculate on the finer scale timing of the radiation of this clade.

Taxonomy

The H3, 18S and 16S trees reveal that in general species defined as *Ledella, Spinula, Bathyspinula,* and *Tindariopsis* do appear to be related and form the subfamily

Ledellinae. However there are a few exceptions that can be explained by factors other than oddities of the tree building methods. The relationship of *Ledella ultima* and species belonging to *Spinula* and *Bathyspinula* is unclear. The Spinulinaes appear to be basal to most *Ledella* species except for *Ledella ultima*. From morphological descriptions of *Ledella ultima,* it is clear it is similar to the other *Ledella* species in some features especially with having a submedial rostra. It is also quite different because of the thickening at the mantel margin and its gut morphology (Figures 4-12 and 4-13). Most of the *Spinula* (except *Bathyspinula calcar*) and *Ledella ultima* share the feature of a highly coiled gut thought to be an adaptation to living at abyssal depths (Allen 1978;1979), however even non abyssal species of *Spinula* have this trait (Figure 4-13). More molecular data from genes that bridge the gap between 16S, which is highly divergent, and the conservative nuclear genes of 18S and H3 may help clarify the relationships between *Spinula* spp., *Ledella ultima* and the other *Ledella* species. A more variant gene is also needed to resolve the larger *Ledella sublevis pustulosa ecaudata* clade. The genus *Tindariopsis* as described by Allen (1986) seems to fit within the Ledellinae, however a species not included in that revision, *Tindariopsis sulcata* appears to be more closely related to Malletidae molecularly and morphologically.

Ledella galathea an abyssal species appears to be outside of the Ledellinae based on 18S and 28S sequences. The 16S primers failed to work on this species perhaps because it was too divergent for the family specific primers to anneal. Additionally, based on preliminary data but also supported by morphology, the species *Ledella acuminata* and *Ledella acinula* fall well outside of the family for 16S and 18S (for *L.*

acuminata). Fresh samples of these species are needed to rule out misidentification or contamination. However, it seems more likely to be misclassification in the case of *Ledella acinula* placing this species in the Ledellinae (Allen 1996) is based on morphological similarity with other problematic species such as *Ledella galathea*. It is less likely to be misidentification because we amplified specimens from lots examined by Allen for his description and redescription of that species.

The two 16S sequences from *Ledella aberrata* also fell outside of the Ledellinae, however one aligned with the male *Ledella ultima* clade and the other a short fragment from a different basin aligned with the species of *Nuculana* . The two sequences did not overlap with each other enough to reasonably compare them and came from different basins so they might be different species. Clearly the potential for one of the sequences being a male form limits our ability to determine if this species still belongs in the Ledellinae. However, as the name suggests the morphology of this species is aberrant to the other Ledella species.

Sampling

DNA was not amplified from some species with a small number of individuals. It is unknown how these species would affect the resulting phylogeny, but based on the morphological affinities we make the following predictions: *Ledella lusitanensis, sandersi, similis and verdiensis* (Allen and Hannah 1989) show morphological affiliation with *sublevis* or *pustulosa* (Allen and Hannah 1989). Thus we would expect these species to align with the major Ledellinae clade. *Ledella solidula* is an important species to add as it shows some similarity to *Ledella galathea*, but also shares a unique trait with

Ledella ultima in that it changes shape with age (Allen and Hannah 1989). Unfortunately all attempts to amplify the limited available samples failed.

Effect of heteroplasmy:

In general, while heteroplasmy complicates the phylogeny, the resulting phylogenies are still informative about the evolution of this group. As with other taxa with heteroplasmy, such as Mytilids (Hoeh et al. 1996), it appears that the relationship between the female and male types of mitochondria within and between species is complex. For *Ledella ultima* the male and female types are quite divergent, but for *Ledella sublevis* they are closely related. There are a number of species where their position in the 16S tree suggested the sequence might be the male type. For *Spinula filatova*, the 18S sequence showed affiliation with other *Spinula*, but the 16S was far outside the family like the male of *Ledella ultima*. For *Ledella parva* and *L. oxira,* it is possible the sequences generated for 16S were from the male mitochondrial type and that is why they did not group with the Ledellinae. The sequences may be like the male form of *Ledella ultima* and fall far outside the family (Chapter 3).

We are just beginning to explore DUI in protobranchs. Using nuclear genes we can support the monophyly of some species of the Ledellinae and use that evidence to determine that the *Spinula filatova* 16S may be from the male type. For other sequences that fall outside the major Ledellinae clade we must be cautious in our interpretation of whether the sequences are from the male form or need to be reclassified. Clearly further analysis needs to be done.

Conclusions

There is a large monophyletic grouping of species classified in the genus *Ledella*. The genus Spinula and *Ledella ultima* are basal to this group. A few species classified as *Ledella* fall outside this clade, but reasonable explanations exist for this. Focusing on the monophyletic Ledellinae clade, the phylogeny provides several important revelations about the evolution of this group. The clade is composed entirely of deep-sea species. The most basal species are the deepest suggesting a deep origin for this group. *Ledella ultima* and the *Spinula* form a deep branch basal to the other confamilial species with a more recent radiation at bathyal depths. It has recently been suggested that the bathyal region is more conducive to population differentiation and speciation resulting the highest species richness (Etter et al. 2005; Rex and Etter 2010). Phylogenetic analysis of the Ledellinae suggests the radiation around 65 mya after the global anoxic event during the Paleocene (Jacobs and Lindberg 1998). The ancient divergence of *Ledella ultima* suggests this family radiated from deep refugia. Assigning and interpreting exact dates of historical events is problematic because of inaccuracies of the molecular clock and the lack of fossil data to properly date divergences, but the resulting phylogenetic topologies some insight into the pattern of evolution and the nature of colonization of the deep Atlantic.

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FIGURE 4-1: Hypothetical relationship between the families and subfamilies within the superfamily Nuculanacea after Maxwell (1988).

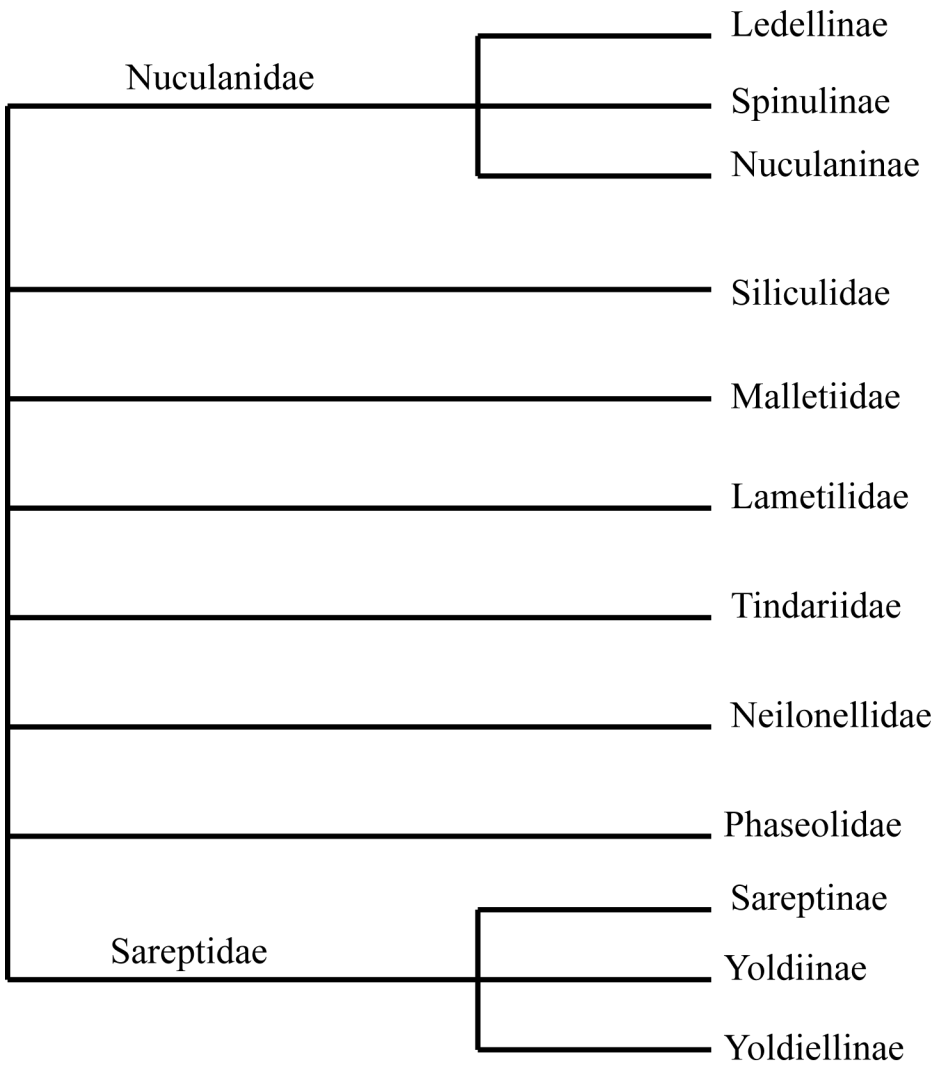

TABLE 4-1: Specimen list with distribution, depth range, source information, and reference for taxonomic description. *Fresh material

Species	Region	Depth	Source	Ref.
Ledella aberrata	ARG/GUY/NAB/WEB/ CAPE	2138-5223m	WHOI	a
Ledella acinula	BRA/GUY/WEB/GUI	943-4632m	WHOI	b (c)
Ledella acuminata	WEB/MED	457-1427	WHOI/SOC	d.(e)
Ledella ecaudata	ANTARCTIC	1000-5000m	ANDEEP*/ MCZ	f.
Ledella galathea	WEB/GUINEA/SIERRA LEONE	4279-4980	WHOI/SOC	g.
Ledella jamesi	ARG/GUY	1456-2853	WHOI/MCZ	d.
Ledella kermadecensis	Pacific	2200-5200m	USNM	g.
Ledella oxira (Nuculana?)	NAB/BRAZIL	678-1493	USNM	d. (h.)
Ledella parva (Nuculana?)	NAB/GOM	450-1180m	USNM	i.
Ledella pustulosa argentina	ARG	3305-5223	WHOI	d.
Ledella pustulosa hampsoni	CAPE VERDI	2051-2357	WHOI	d.
Ledella pustulosa marshali	WEB	2466-4632	SOC/GAGE*	d.
Ledella pustulosa pustulosa	WEB/CAN	609-2659	SOC/GAGE*	d. (j.)
Ledella sublevis	Atlantic Cosmopolitan	2022-4680	WHOI/EN147 *	k.
Ledella ultima	Cosmopolitan	3196-5130	WHOI/SOC/ EN147*	(l)
Ledella sp. (M2751)	Pacific Peruvian trench	3475m	SCRIPPS	
Bathyspinula sp.	Pacific		USNM	
Spinula filatovae	Angola/WEB/Cape Verde/Gui	1261-4340m	BMNH*	m.
Bathyspinula calcar	Pacific	4067-5535m	SCRIPPS	(n) f.
Spinula hilleri	ANTARCTIC/Atlantic wide	2231-5227m	ANDEEP	b.
Spinula scheltemae	WEB	1125m	SOC	b.
Spinula subexcisa	WEB	1993-3020m	SOC	b. (o)
Ledella (Tindariopsis) agatheda		1942-2076m	MCZ	a. (c.)
Tindariopsis sulculata			BMNH	(p)
Nuculana fossa	Pacific			
Nuculana inaequisculpta			BMNH	
Nuculana minuta	Skagerat 102		Skagcruise*	
Nuculana pernula	Skagerat 132		Skagcruise*	
Propeleda longicaudata			ANDEEP*	

a. Allen and Sanders, 1996 b. Allen and Sanders, 1982 c. Dall 1890 d. Allen and Hannah, 1989 e. Jeffreys, 1870 f. Filatova & Shylecko,1984 g. Knudsen, 1970. h. Dall, 1927 i. Verrill and Bush 1897 j. Jeffreys, 1876 k.Verrill and Bush 1898 l. Smith 1885 m. Knudsen 1967 n. Dall 1908 o. Dautzenberg and Fischer, 1897 p. Gould 1852

TABLE 4-2: Primer list

Primer name	Sequence 5' to 3'	Reference
General primers 16S Nuculanidae		
16Sar	ATG TTT TTG ATA AAC AGG CG	Kocher *et al*. 1989
Lu16R4	GCT GTT ATC CCT CCA GTA AC	Chase *et al*. 1996
Led16Alt2F	CCG TYC AAA GGTAGT GTA AT	This study
LANA316RB	AGC TCA GTT GCC CCA ACT AAA	This study
18S primers – protobranch specific		
Proto18sA2f	ATGCATGTCTAAGTACANACT	This study
Proto18bb2r	AACCACGGTAGGCATATCA	This study
28S primers		
28sa	GACCCGTCTTGAAACACGGA	Giribet 2006
28SRA	GAA AAG ARA ACT CTT CCC GG	Chase unpublished
H3 primers		
H3F	ATGGCTCGTACCAAGCAGACVGC	Colgan et al. 2000
H3R	ATATCCTTRGGCATRATRGTGAC	Colgan et al. 2000

TABLE 4-3: Summary of sequences for Ledellinae and Nuculanidae. For 16S and 28S arrows indicate relative position of primers used. * Species appears to be in the genera Ledella. ** For 18S and H3 the same primers were used for all individuals the size of the fragment is indicated.

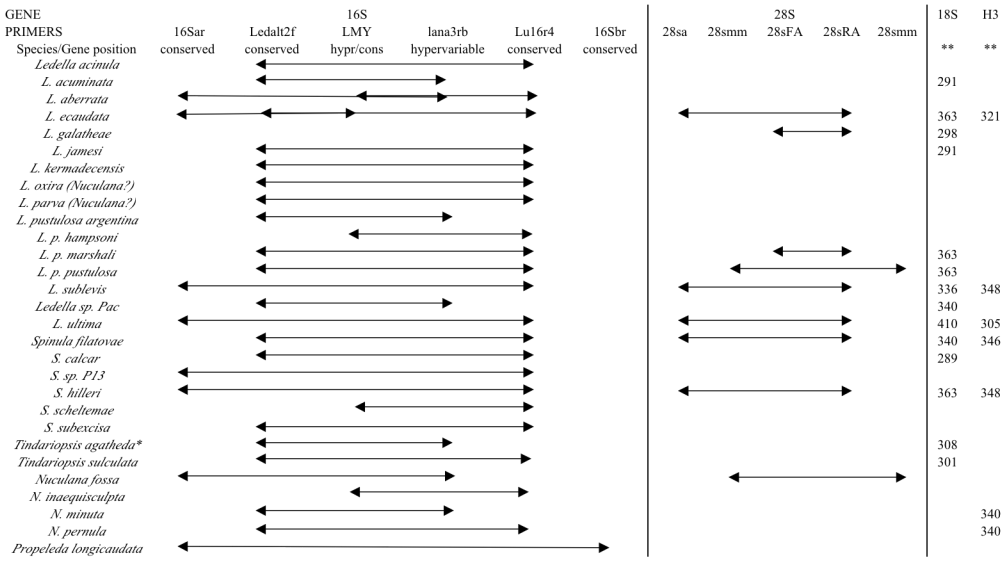

TABLE 4-4: Summary species and sequences for Protobranch phylogeny. For 16S and 28S arrows indicate relative position of primers used. For 18S, H3 and COI the same primers were used for all individuals the size of the fragment is indicated.

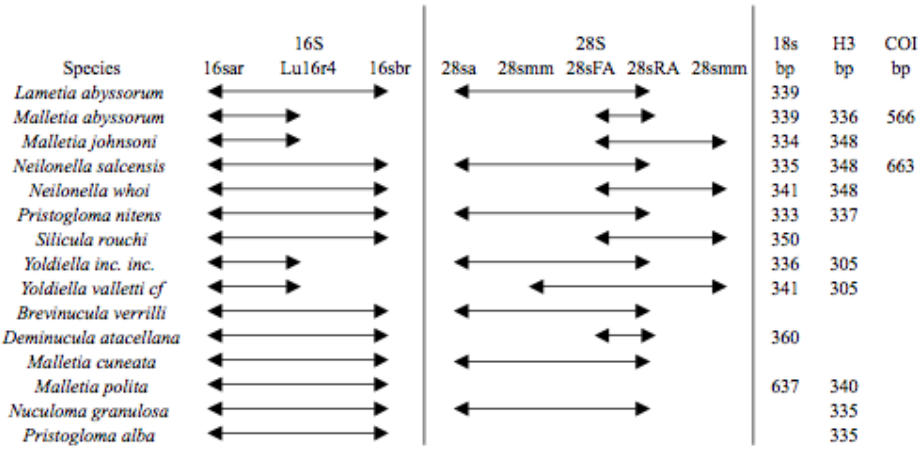

FIGURE 4-2: Bayesian tree based on a portion of 18S. Branch labels are posterior probabilities. The Ledellinae clade is highlighted in red.

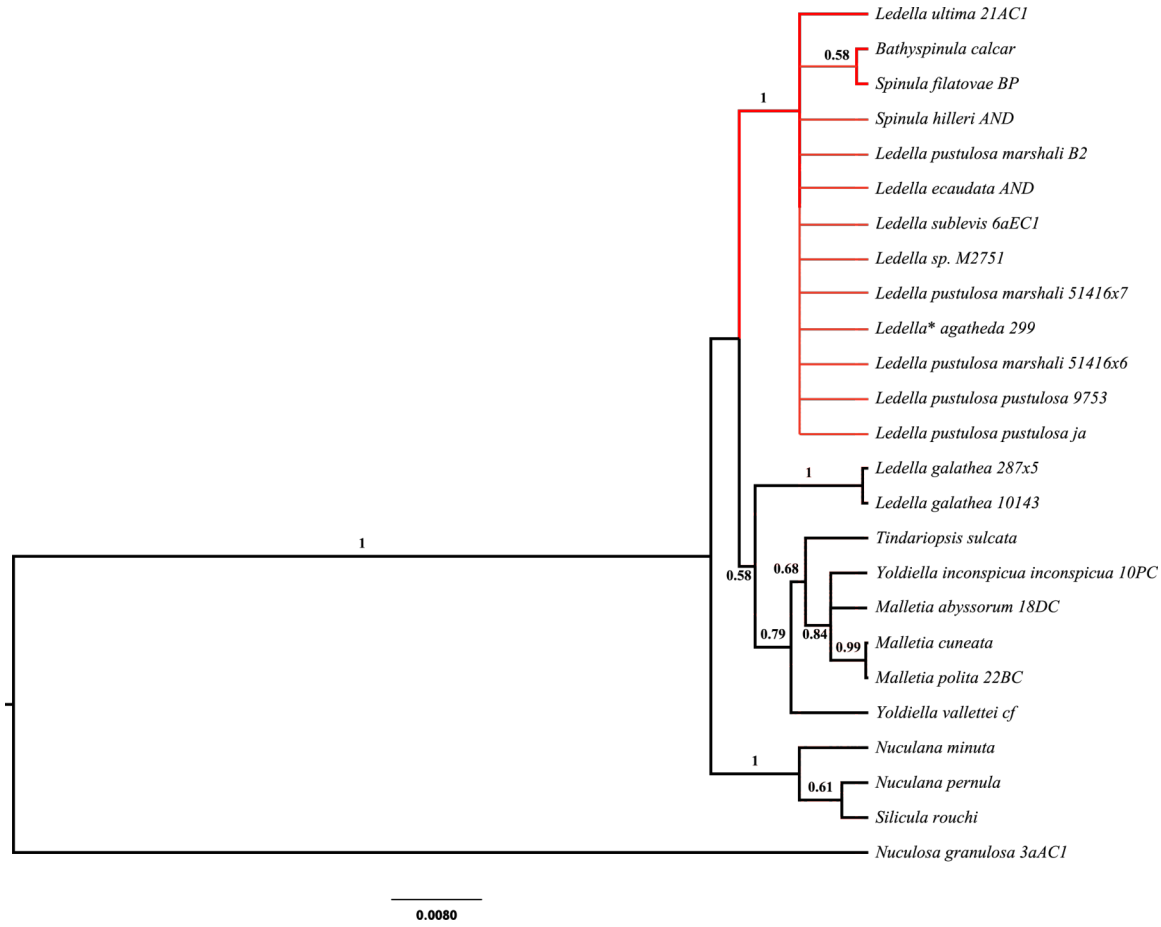

FIGURE 4-3: Bayesian tree based on a portion of H3. Branch labels are posterior probabilities. The Ledellinae clade is highlighted in red.

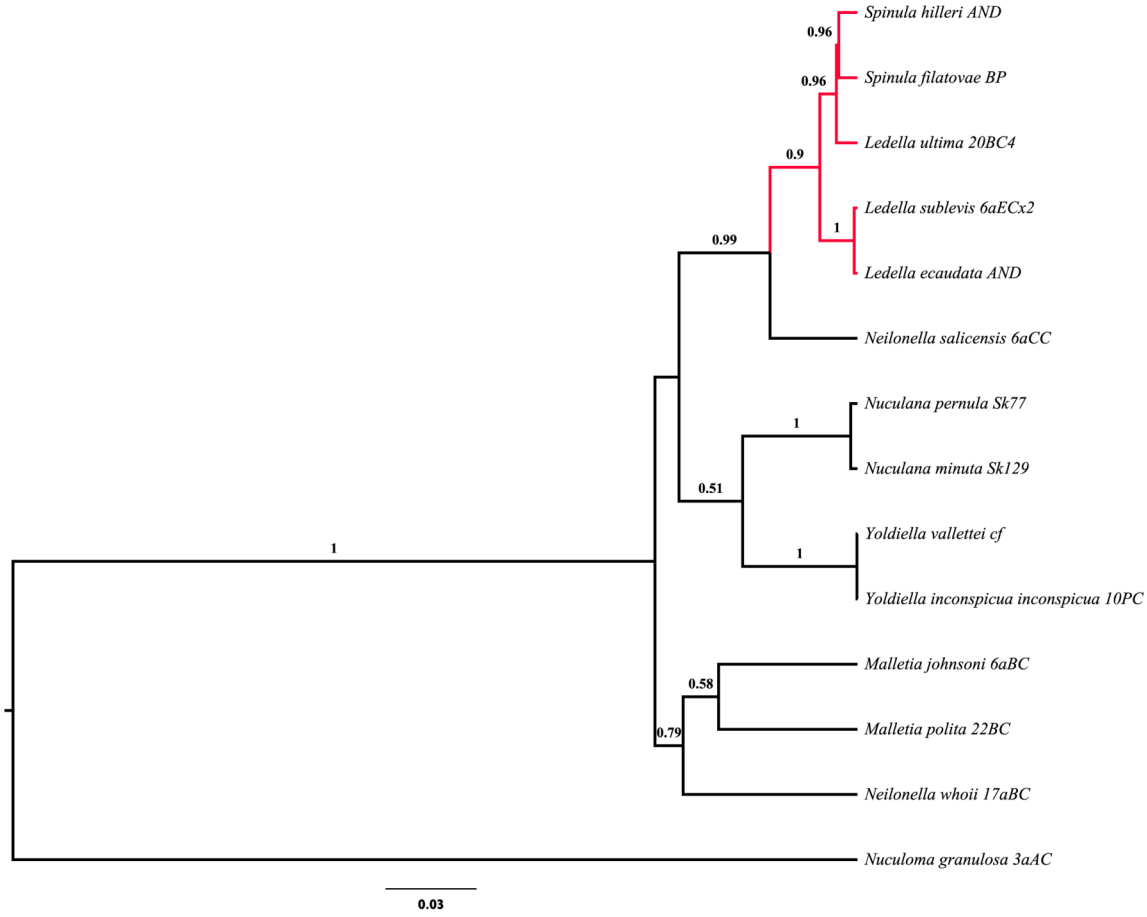

FIGURE 4-4: Bayesian tree based on a portion of 28S. Branch labels are posterior probabilities. The Ledellinae are highlighted in red.

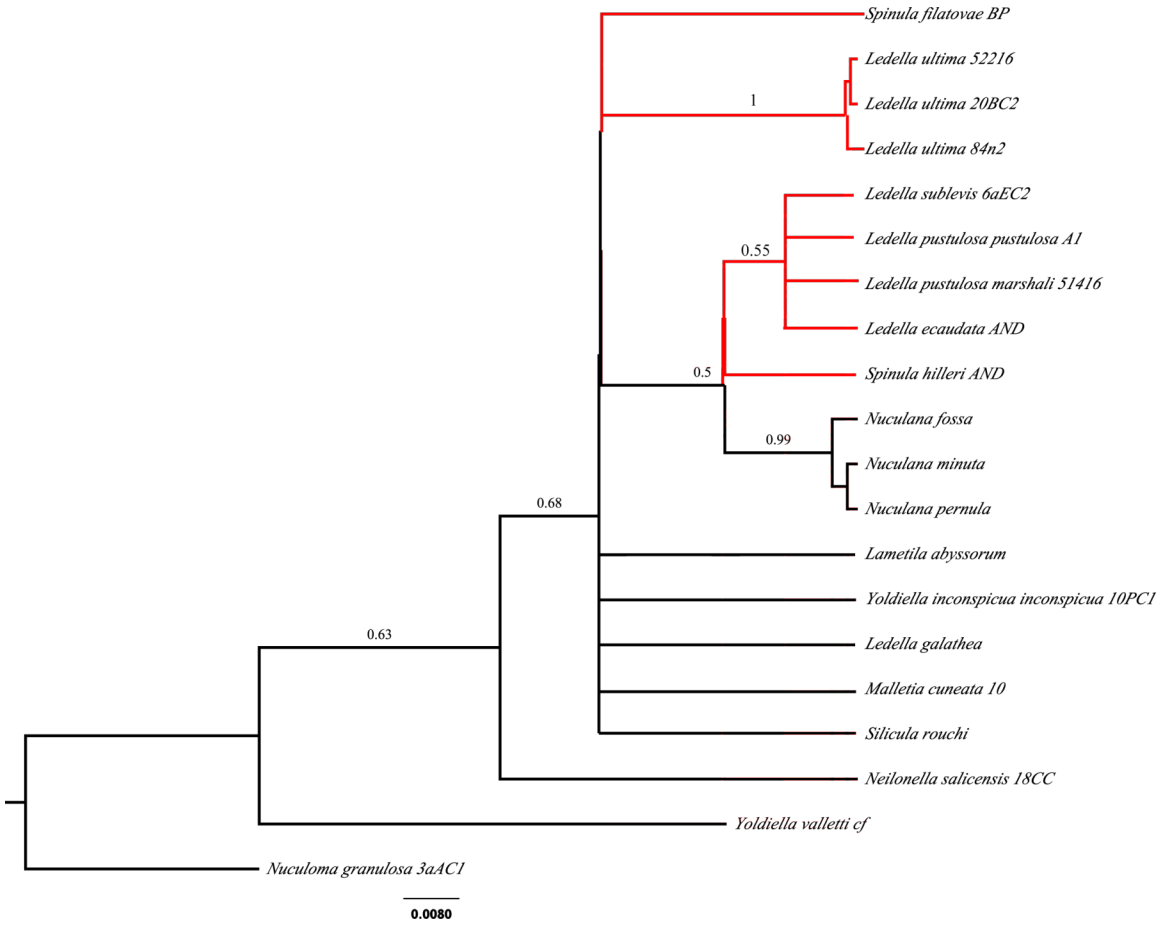

FIGURE 4-5: Bayesian tree of combined H3/18S/28S data. Posterior probabilities are indicated on branches. The Ledellinae clade is highlighted in red.

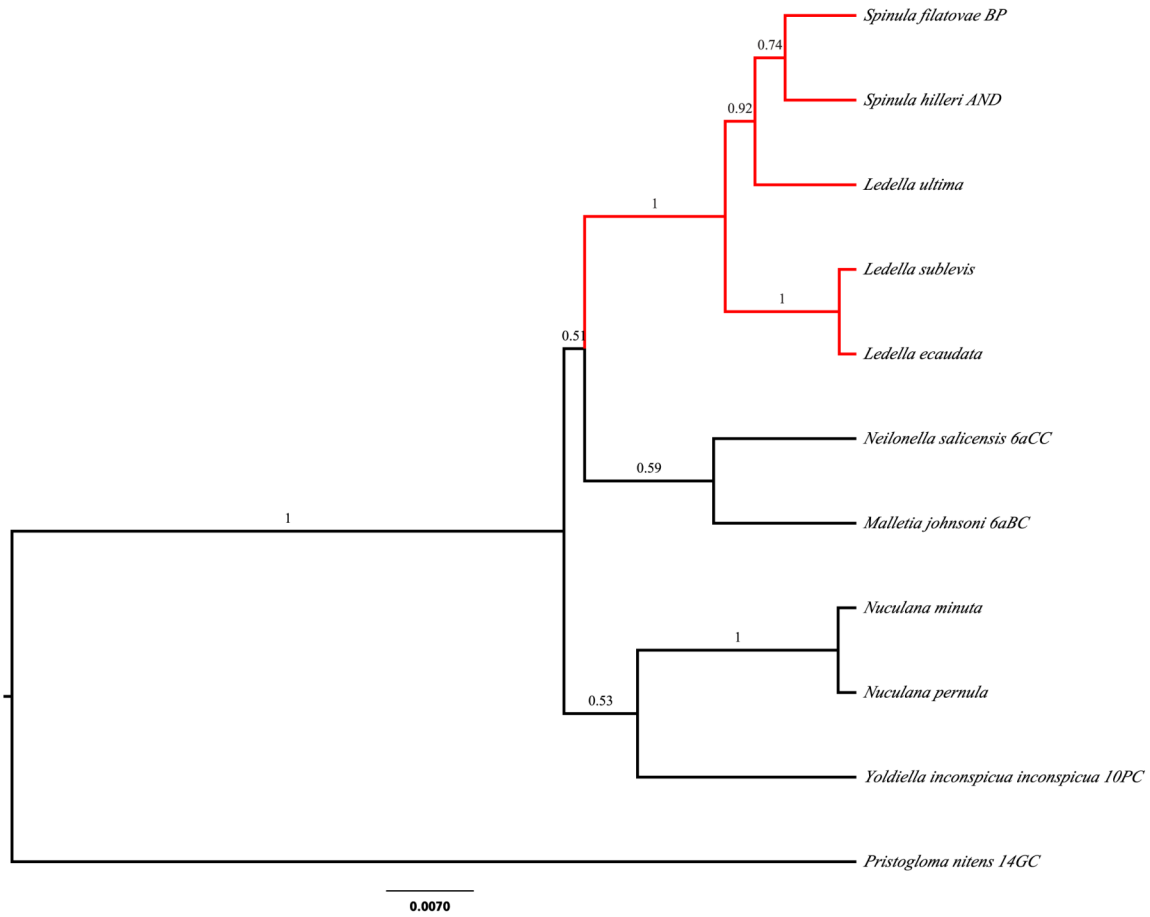

FIGURE 4-6: Bayesian tree of 16S gene sequences includes all samples including known and suspected male haplotypes (highlighted in blue). Posterior probabilities are indicated on branches. The Ledellinae clade is highlighted in red. Number next to collapsed clades indicates the number of sequences in that clade.

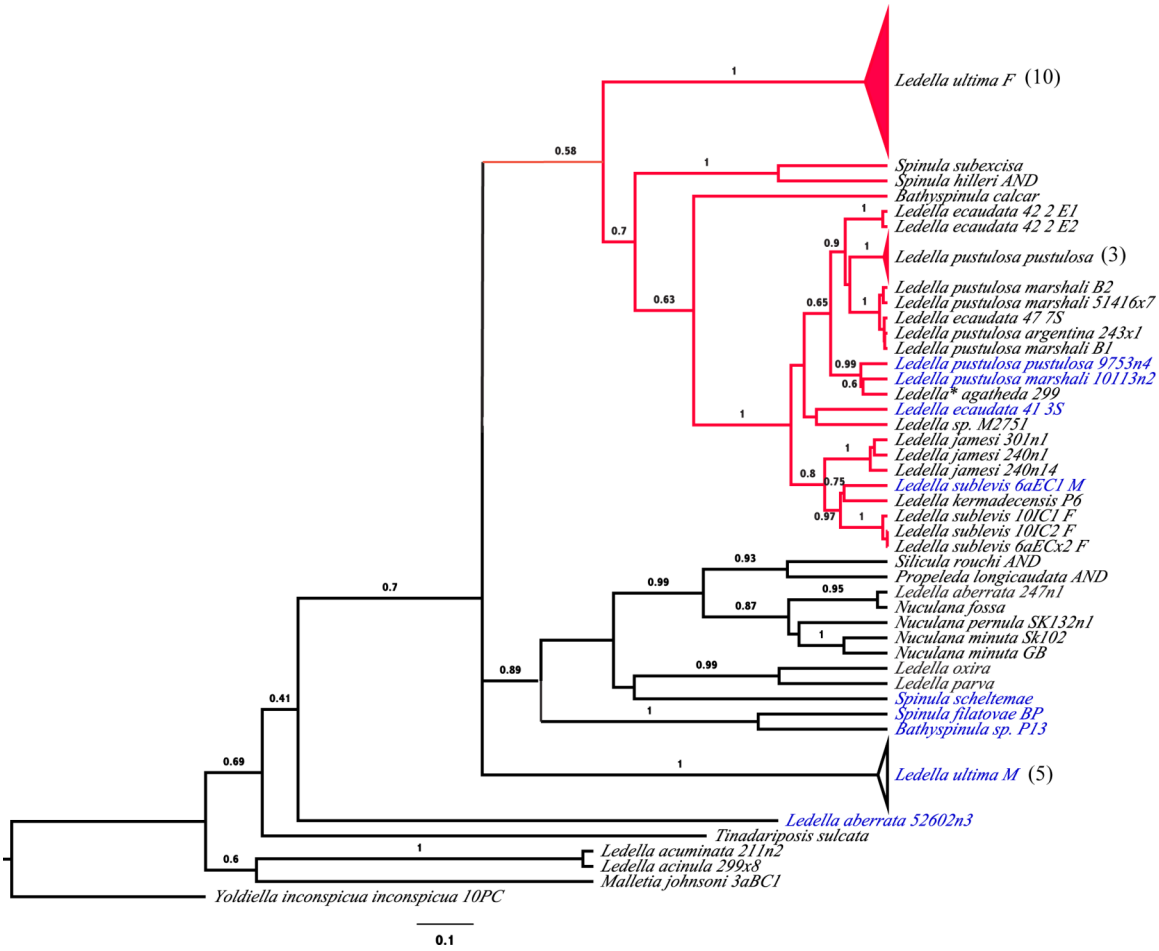

FIGURE 4-7: Bayesian tree of 16S gene sequences includes all 16S without suspected male haplotypes. Posterior probabilities are indicated on branches. The Ledellinae are highlighted in red.

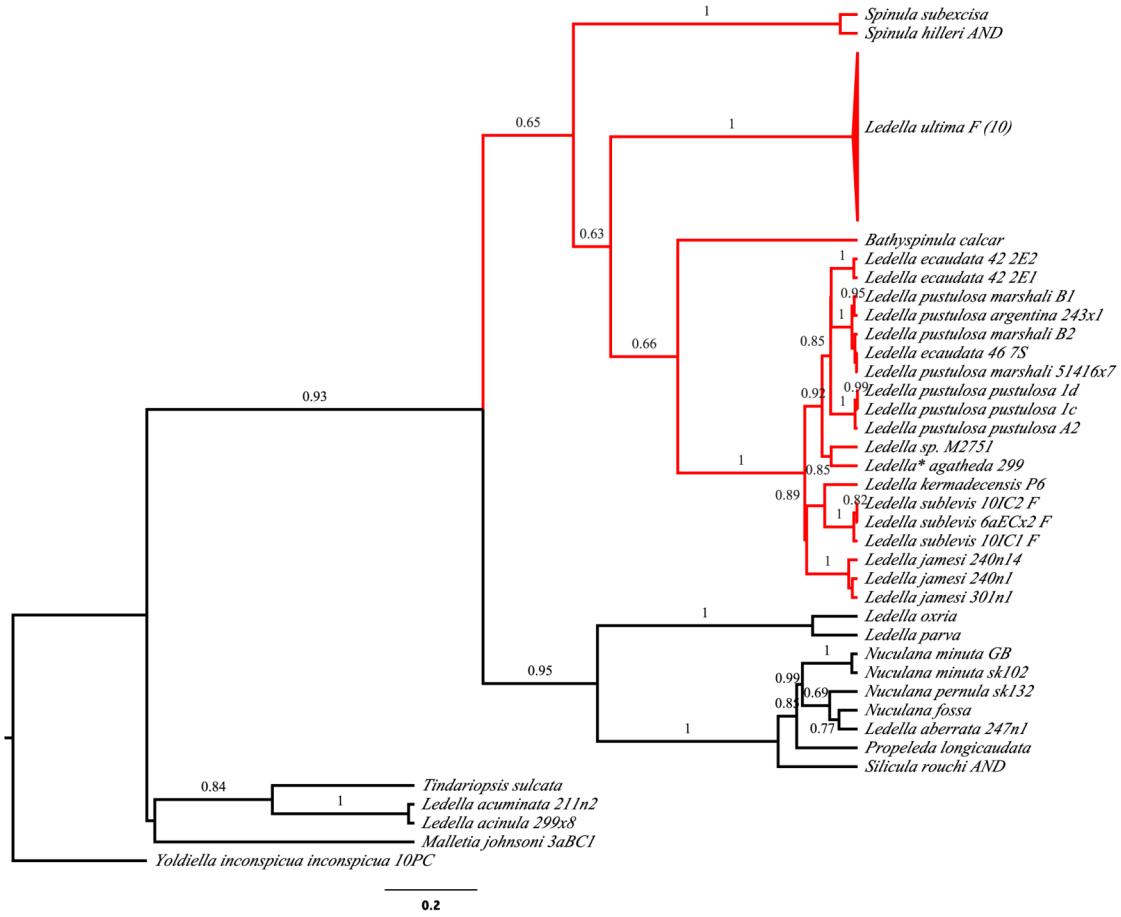

FIGURE 4-8: Bayesian tree of 16S gene sequences includes all 16S without suspected male haplotypes and short sequences removed. Posterior probabilities are indicated on branches. The Ledellinae clade is highlighted in red. Number next to collapsed clades indicates the number of sequences in that clade.

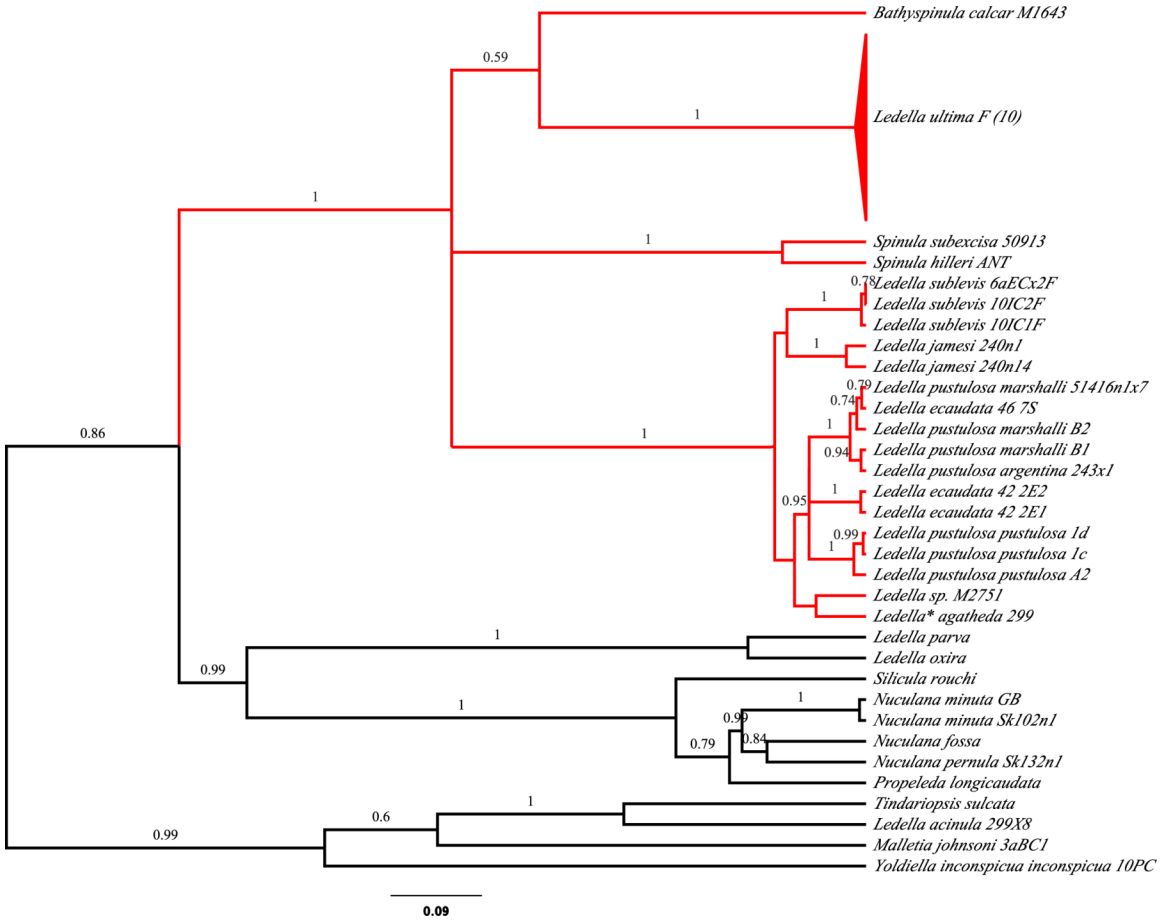

FIGURE 4-9: Bayesian tree of 16S gene sequences includes all 16S including suspected male haplotypes and short sequences removed. Posterior probabilities are indicated on branches. The Ledellinae clade is highlighted in red. Number next to collapsed clades indicates the number of sequences in that clade.

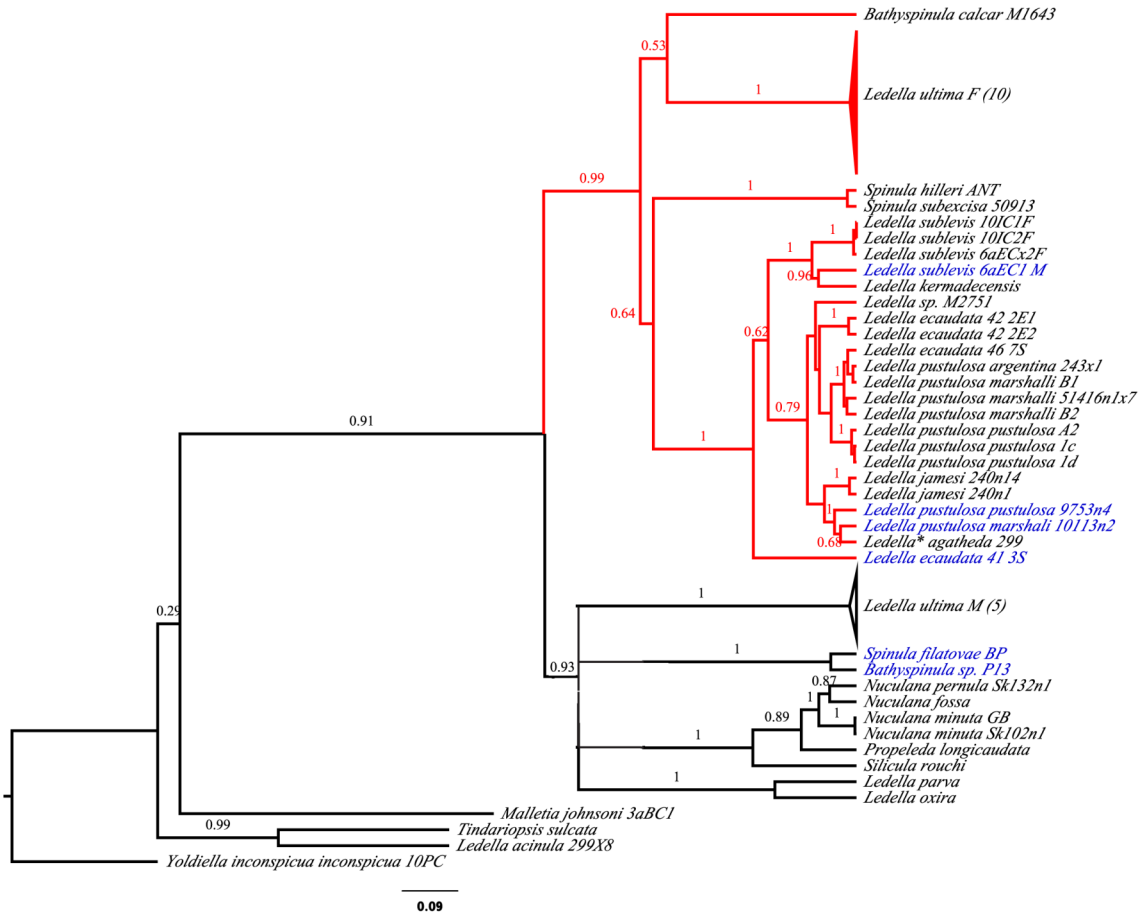

FIGURE 4-10: Bayesian tree of combined 18S and 16S data. Posterior probabilities are indicated on branches. The Ledellinae clade is highlighted in red.

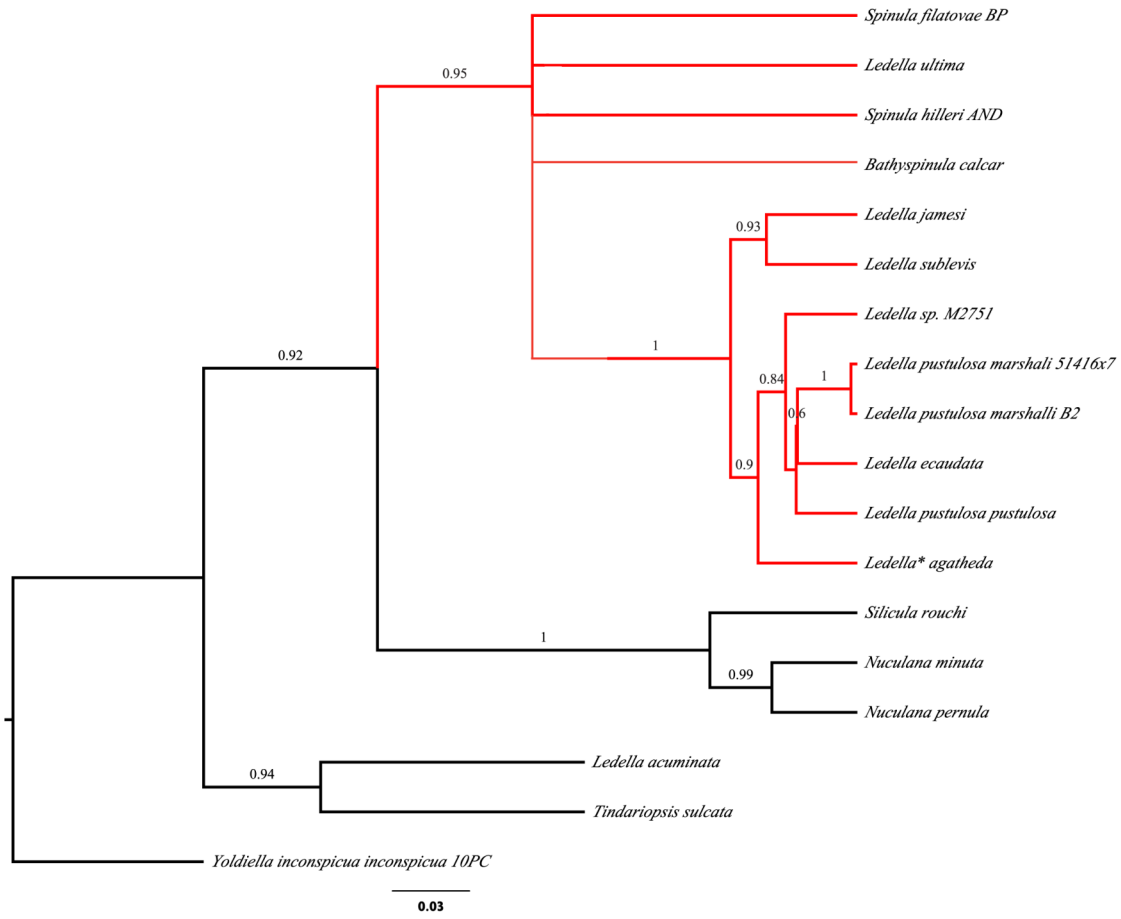

FIGURE 4-11: Geographically referenced Bayesian tree based on 16S alignment with molecular clock indicated in millions of years. Posterior probabilities are indicated on branches. The Ledellinae clade is highlighted in red. Color of species indicates geographic locality as described in the legend.

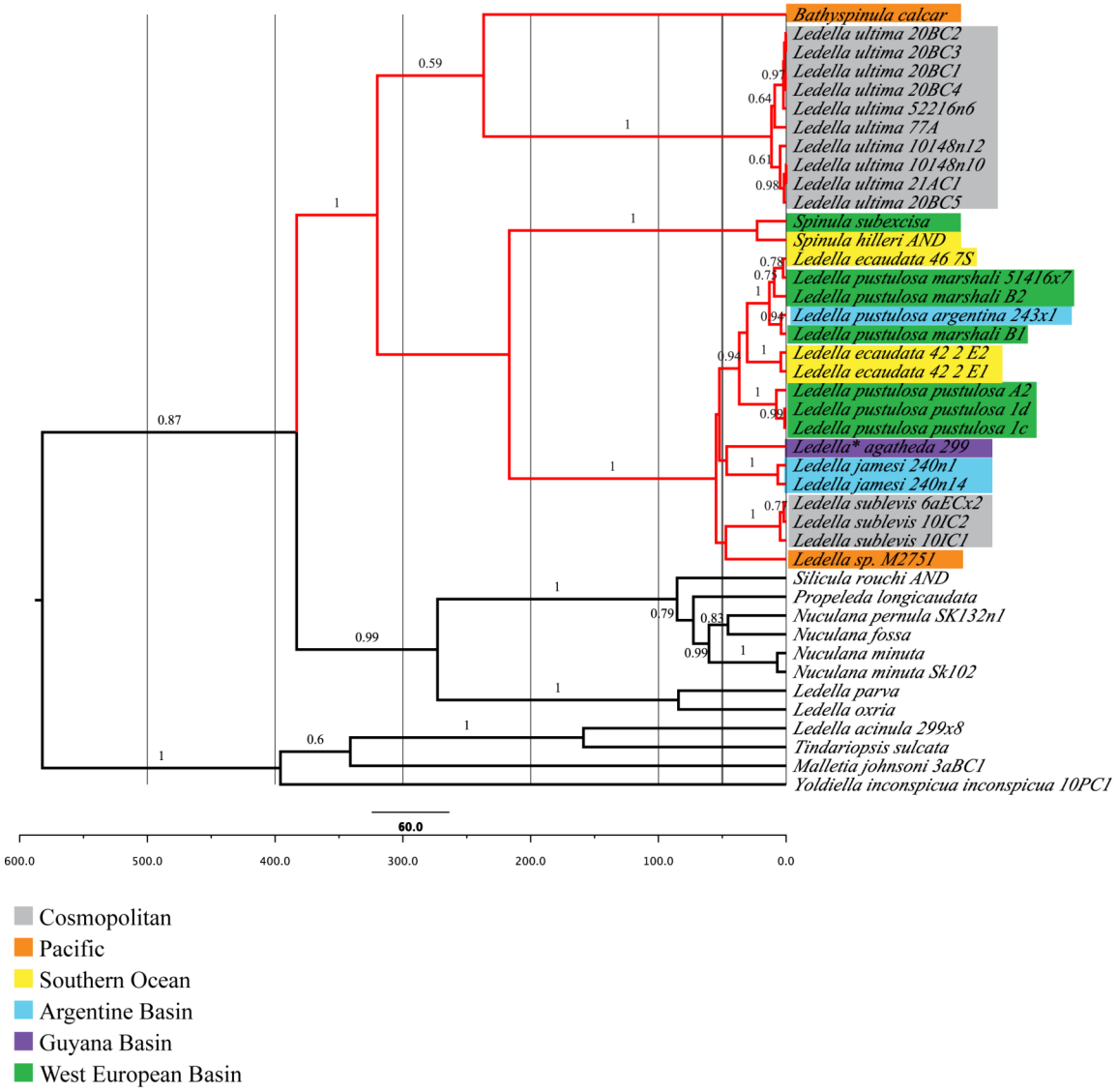

FIGURE 4-12: Dorsal view of *Ledella* species (Allen and Hannah, 1989) and *Spinula* species (Allen and Sanders, 1996).

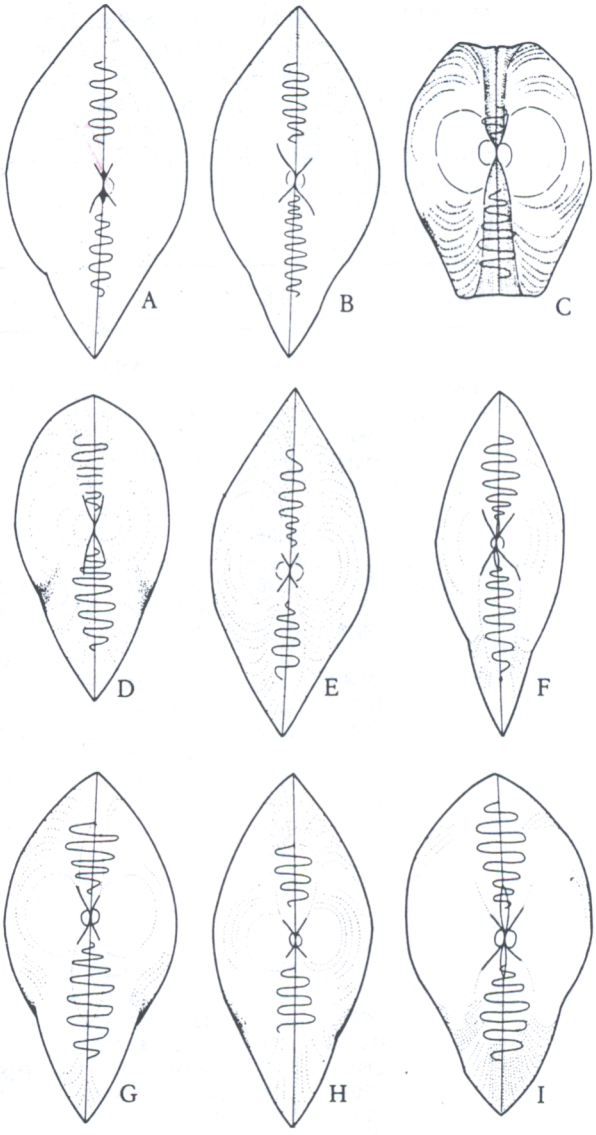

Fig. 3 Dorsal views of the shells of various species of *Ledella* for comparison. *A, Ledella pustulosa marshalli*; B, *L. pustulosa pustulosa*; C, *L. ultima*; D, *L. lustanensis*; E, *L. pustulosa argentinae*; F, *L. acuminata*; G, *L. sublevis*; H, *L. jamesi*; I, *L. solidula*.

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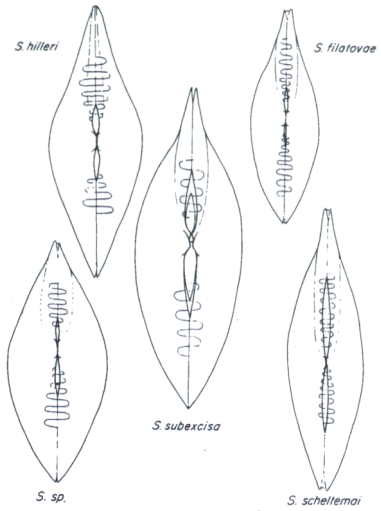


Figure 28. Dorsal view of shell of the five species of *Spinula* from the Atlantic to show differences in shell shape and extent of external ligament.

FIGURE 4-13: Hind gut morphology of *Ledella* (Allen and Hannah, 1989) and *Spinula* (Allen and Sanders 1996) species

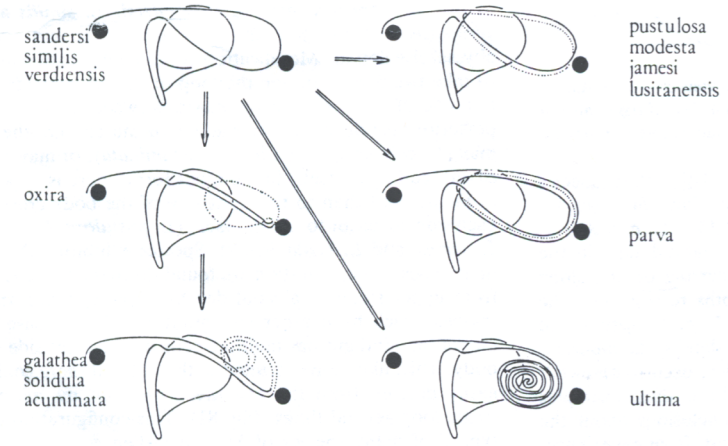
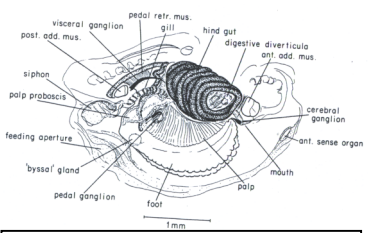
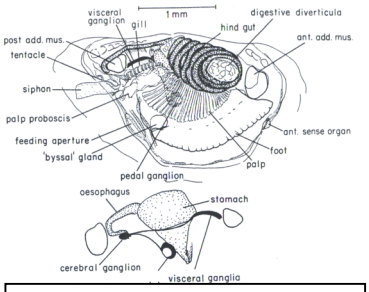


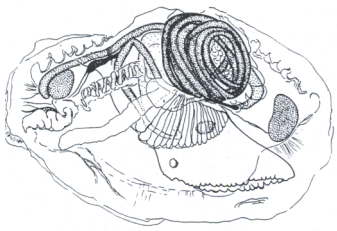
Fig. 81 The radiative evolution of hind gut configurations in species of *Ledella*.



Spinula hilleri



Spinula subexcisa



Ledella ultima

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